AMERICAN ASSOCIATION OF CEREAL CHEMISTS

Monograph Series **VOLUME I**

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ENZYMES
and Their Role in Wheat Technology

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PREFACE

Preparation of a series of monographs, of which this is the first volume, is sponsored by the American Association of Cereal Chemists primarily as a service to its members, but the series should prove useful to many other persons. The task of keeping abreast of the advances in knowledge recorded in the scientific journals is becoming increasingly difficult, especially for those like cereal chemists who must strive to apply results obtained in several branches of science. Indeed, in many active fields of investigation, papers appear so frequently that one man, even though he has ready access to a wide range of scientific periodicals, can hardly hope to find and read all that is published, much less to co-ordinate the information and summarize it for others in a book.

The monograph, in which a number of experts write chapters on related topics, provides a means of overcoming these difficulties. From the reader's point of view, and also from the editor's, such books present their problems. With several authors involved, there must be considerable variety in the treatment of parallel topics, and a certain amount of overlapping is difficult to avoid. But what is lost in these respects is offset by the authority that stems from having each chapter written by a specialist.

Enzymes were chosen as the subject for the first volume in this series because of the important part they play in wheat technology and the rapid progress that has recently been made in our knowledge of them. Aside from the introductory chapter, the treatment has been restricted to the enzymes of greatest interest to cereal chemists: amylases, proteases, lipases, oxidases, and the fermentation enzymes. There are twin chapters for each of these classes. The first contains a broad review of existing knowledge of the class, and covers enzymes of both plant and animal origin; the second presents a discussion of what is known of the part played by the enzymes in wheat technology. Those who seek inspiration for further investigations and applications in the more practical fields considered in the second of each pair of chapters may well find it in the first.

The Monograph Committee was appointed by the Association early in 1942 with Dr. W. F. Geddes as chairman and the following members: Drs. C. H. Bailey, E. G. Bayfield, M. J. Blish, F. C. Hildebrand, and R. K. Larmour. A year later, the writer joined the committee to replace Dr. Larmour, and in September of 1943, after Dr. Geddes had become editor-in-chief of *Cereal Chemistry*, the writer was asked to take the chairmanship of the Monograph Committee and the responsibility for editing Volume I.

The American Association of Cereal Chemists is deeply indebted to the authors who contributed to this volume. With increased responsibilities and the press of more important work, it has not been easy to find time for writing. Grateful acknowledgments are also made to the members of the Monograph Committee, to the publishers, and to many others who have contributed unselfishly of their time and ability, particularly Miss Kathleen Webb, Assistant Editor of *Cereal Chemistry*, who undertook the exacting task of reading galley and page proofs.

For myself, I have made many valuable associations, and have appreciated the cordial relations with so many able chemists that the editing of this monograph has made possible. J. A. A.

Winnipeg, Manitoba
February, 1946

Enzymes and their role in wheat technology

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CHAPTER I

THE GENERAL CHEMISTRY OF ENZYMES

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CATALYSIS AND ENZYMES

Most of the chemical changes that occur in living tissues are regulated by biocatalysts, the enzymes. A catalyst is a substance that alters the speed of a reaction without appearing as part of the final products and without changing the energy content of the reactants and resultants. According to this definition a catalyst merely alters the rate of a reaction already in progress. Some investigators insist, however, that a catalyst may initiate a reaction.

Ostwald proposed three criteria for the ideal catalyst: (1) The substance should be returned unchanged chemically at the end of the reaction, though it may be altered physically. Thus the crystalline pyrolusite used to aid in the liberation of oxygen from potassium chlorate finally appears as an amorphous powder. (2) The catalyst should function in small quantities, and the amount of change should be proportional to the quantity added. (3) The catalyst should not change the equilibrium point of the reaction. As a corollary, it follows that the same catalyst should function whenever the equilibrium point can be approached from both sides; and this has been verified in many cases. There are instances where an apparent shift in the equilibrium point can be accounted for by a combination of one of the components with the catalyst. In the reaction, ethyl acetate + water $\rightleftharpoons$ ethyl alcohol + acetic acid, in the presence of organic acids, it was shown by Kendall and Booge (1916) that the catalyst formed an addition compound with part of the ester; but, when the appropriate calculations were made, the equilibrium point was found to be constant irrespective of the side from which it was approached.

Another hypothesis states that the catalyst cannot initiate a reac-

tion. This is a moot question that is not readily answered by experiments because it is no easy task to demonstrate that the reaction is not proceeding too slowly to yield a measurable change. It is also difficult to devise conditions under which the presence of a catalyst may be ruled out, for it is known that such agencies as traces of moisture, dust particles, the solvent, and the walls of the containers may influence the course of a reaction. It is also recognized that the catalyst may combine with one of the reactants to form a new and unstable substance; and there may thus arise a cyclic process which may cause the initiation of an entirely new trend in the reaction mixture.

Most catalysts have a well-defined specificity. Thus carbon monoxide and hydrogen are converted to methane and water almost quantitatively when passed over nickel; but the same compounds yield methyl alcohol quantitatively in the commercial process using a mixture of zinc and chromic oxides; whereas, with alkali and iron, a mixture of higher alcohols, ketones, and hydrocarbons is obtained.

Catalysts permit reactions to occur under milder conditions than would otherwise be possible, and the enzymes illustrate this to a noteworthy degree. The average protein is hydrolyzed by boiling with 20 per cent hydrochloric acid for 24 hours at a temperature of about 108°C; on the other hand, by enzymatic action in the higher animals, proteins are digested within 4 hours at a temperature of only 38°. If the rule that the average rate of reaction is doubled with each 10° rise in temperature is assumed, and if account is taken of the time periods involved, the enzyme reaction appears to proceed over seven hundred times more rapidly than the acid hydrolysis. Obviously no allowance is here made for the quantity of catalyst involved.

Enzymes are catalysts. They may be defined as thermolabile catalysts of a definite organic nature, elaborated by living tissues but capable of action outside the latter. The vitamins and auxins are excluded because these compounds are thermostable and perform their physiological roles only in living matter. The phrase "of a definite organic nature" is in part assumed since the structures of only a few enzymes are known, but it is added to exclude surface phenomena.

The enzymes are found in or near the tissues where they are to be used. In the economy of nature, the wheat berry functions to provide the young plant with nutrients until it is self-sustaining; hence it contains immobile reserves of proteins, carbohydrates, and fats, together with the enzymes or their precursors necessary to convert these into soluble metabolites. Similarly, in man and the higher animals, the juices emptying into the alimentary tract contain the enzymes necessary to render foods soluble before absorption. There are exceptions to this generalization;

for example, the jack bean is notably rich in urease, whereas urea plays little if any part in the nitrogen metabolism of this plant.

CLASSIFICATION AND NOMENCLATURE

The enzymes, or the "ferments" of the older literature, are named systematically by adding the suffix "ase" to the stem of the name of the substrate. Thus amylase and lactase are generic names for the enzymes acting upon amylose (starch) and lactose. To designate the particular source of the enzyme one may refer, for example, to a malt or a pancreatic amylase. The word invertase is an exception since it designates the enzyme producing invert sugar from sucrose; this enzyme is also called sucrase or saccharase. Such names as pepsin, rennet, and trypsin were used before a system of nomenclature was adopted, and they are retained. A single name is sometimes retained for a specific group of enzymes which occur together in nature and which catalyze an over-all change through the activities of the constituent enzymes acting in series. The trypsin fraction of the pancreas has been fractionated to yield chymotrypsin, trypsin proper, and carboxypeptidase as discrete proteases (Northrop, 1939).

Two large classes of enzymes (Table I) are distinguished by the reactions they catalyze: the hydrolases, which cleave linkages with the addition of water; and the desmolases, which break linkages not attacked by water. The reactions catalyzed by the latter often involve ruptures of an oxygen molecule or of a carbon chain. The roles played by coagulating enzymes, such as rennet and thrombin, are not well understood. Perhaps these constitute a distinct group, but at present they are considered as hydrolases.

In the older literature a distinction was made between the organized and the unorganized ferments, which are now called the intracellular and the extracellular enzymes. It was believed that the intracellular enzymes could function only within the organized tissues whereas the extracellular enzymes were those secreted or readily extracted. This distinction was removed when Büchner (1897) demonstrated that zymase, which was held to be an organized ferment, could function even though every yeast cell was ruptured. The differences are of degree and are of significance only when an extraction of the enzyme is to be made.

At times an enzyme precursor (proenzyme) is found in nature. This inactive state is known as the zymogen. For example, pepsinogen is elaborated by the membranes lining the pyloric end of the stomach, and is rapidly converted into pepsin by the hydrochloric acid from the fundus region. Both pepsinogen and pepsin have been obtained in the crystalline state and have been shown to differ in several respects (Northrop, 1939);

and this rules out the possibility that the observed activity was caused merely by a change in hydrogen-ion concentration. Often the activating agent is an organic substance such as the enterokinase secreted by the intestinal walls, which can convert trypsinogen and procarboxypeptidase

TABLE I

A CLASSIFICATION OF ENZYMES

Enzyme	Substrate	End-products
I. Hydrolases		
A. Esterases		
1. Lipases		
a. Glyceridases	Glycerides	Glycerol + fat acids
b. Lecithinase	Lecithin	Glycerol + choline + H_3PO_4 + fat acids
2. Choline esterases	Acetylcholine	Choline + acetic acid
3. Chlorophyllase	Chlorophyll	Phytol + chlorophyllide
4. Phosphatases		
a. Phosphomonoesterases	Phosphoric monoesters	Alcohol + phosphoric acid
b. Phosphodiesterases	Phosphoric diesters	Monoester + alcohol
c. Phytase	Phytin	Inositol + phosphoric acid
B. Carbohydrases		
1. Disaccharidases		
a. Invertase	Sucrose	Glucose and fructose
b. Maltase	Maltose	Glucose
c. Emulsin	β-Glucosides	Glucose and prosthetic group
d. Cellobiase	Cellobiose	Glucose
e. Lactase	Lactose	Glucose and galactose
2. Polysaccharidases		
a. Amylases	Starch	Maltose and dextrins
b. Cellulase	Cellulose	Cellobiose
c. Cytase	Hemicellulose	Sugars
d. Phosphorylase	Glycogen	Glucose-1-phosphoric acids
C. Proteases		
1. Proteinases	Proteins	Amino acids and peptides
a. Trypsin		
b. Pepsin		
c. Papain		
2. Protein coagulases		
a. Rennet	Casein	Paracasein
b. Thrombin	Fibrinogen	Fibrin
3. Polypeptidases	Polypeptides	Amino acids
a. Aminopeptidases		
b. Carboxypeptidases		
4. Dipeptidases	Dipeptides	Amino acids
5. Desamidases		
a. Urease	Urea	Ammonium carbonate
b. Asparaginase	Asparagine	Ammonia and aspartic acid
c. Arginase	Arginine	Urea and ornithine
II. Desmolases		
1. Zymase	Hexose phosphate	Carbon dioxide, ethanol, and phosphate
2. Catalase	Hydrogen peroxide	Water + oxygen
3. Peroxidase	Hydrogen peroxide + reducing agent	Oxidized product
4. Carbonic anhydrase	Sodium bicarbonate	Carbon dioxide + sodium hydroxide
5. Oxidoreductase systems		
a. Direct oxidases		
(1) Phenolases	Phenols	Quinone
(2) Uricase	Uric acid	Allantoin + carbon dioxide
b. Dehydrogenases (oxidoreductases)	Various metabolites	Oxidized (dehydrogenated) products
c. Mutases	Aldehydes	Alcohol + acid

(Northrop, 1939) into the active form. In some instances the occurrence of a zymogen may be confused with the state in which the active enzyme is temporarily protected or "blocked" in its action by other materials, chiefly proteins.

The enzyme proper is often insufficient to catalyze a reaction without the aid of specific substances, such as the activators or the coenzymes. In general, the coenzymes are necessary to obtain any action from certain specific enzymes, whereas the activators are desirable because they give acceleration to the reaction catalyzed. The general term, holoenzyme, is used to indicate a complete enzyme system which consists of the apoenzyme, the thermolabile enzyme proper, plus the dialyzable coenzyme. With zymase, a third term, atiozymase, is employed to designate the magnesium- and cocarboxylase-free apozymase. There is no complete agreement as to the use of such terms as activator, accelerator, complement, kinase, and coenzyme; the more common usages are here described.

Coenzymes are usually considered as the dialyzable, heat-stable portion of certain enzyme systems; and they are of equal importance with the enzymes proper. Harden and Young (1906) demonstrated that a certain fraction of yeast could be removed by dialysis, and that neither it nor the residue would cause alcoholic fermentation, but that the activity was restored upon mixing the two fractions. This phenomenon is more frequently encountered in the oxidizing and fermenting enzyme systems. The pyrophosphate esters of thiamine and of riboflavin are typical examples of coenzymes. These are necessary for certain steps of many oxidations; they dialyze readily, and are not denatured by heat as is the apoenzyme (the enzyme proper).

The term kinase is used to designate an organic molecule that converts a zymogen into an active enzyme but may not be necessary after that transformation has been effected. The best example is the enterokinase that converts trypsinogen into trypsin (Kunitz, 1939).

Complement is a special term used by Pringsheim (Pringsheim and Beiser, 1924) to designate the product that permits amylase to convert starch completely to reducing sugars instead of stopping at the 60 per cent stage obtained with a purified enzyme. It is a peptone and may be identical with the eleutoamylase of Chrzaszcz and Janicki (1936).

The terms activator, stimulator, and accelerator appear to be used synonymously. The increased rate of activity may vary from a few to several hundred per cent. Sodium chloride appears to be essential to the functioning of animal amylases, whereas bromides, iodides, nitrates, chlorates, thiocyanates, and fluorides are less effective with pancreatic amylase (Sherman, Caldwell, and Adams, 1928). These effects do not depend upon the purity of the amylase. Helferich and Schmitz-Hillebrecht (1935) reported an enhancement in emulsin activity of over 300 per cent with thiocyanates and chlorates. Papain and bromelin are activated by hydrocyanic acid and by hydrogen sulfide. Scott and Sandstrom (1942) have shown that mercaptans are much more effective in activating papain

than is hydrogen sulfide, whereas no organic cyanide is effective. An interesting example of the sulfhydryl activation of papain was made by Purr and Russel (1934). They found that some constituent of the blood cells of animals could activate papain *in vitro* and that this constituent was present in cancerous subjects in only half the normal concentration.

In most cases no very satisfactory explanation has been advanced to account for the whole phenomenon so similar to the "promoter" action of inorganic catalysts. Some suggestions are noted here: (1) The activator may act as an emulsifier. Lipase is found in the water phase whereas the oil substrate is in a separate phase. The action is limited to the interface developed, which is greatly increased by such fat emulsifiers as calcium salts, bile salts, and proteins. (2) The activator may peptize the enzyme or the protein carrying the enzyme and thus produce more effective surface of the catalyst. (3) The activator for keratinase is known to act upon the substrate. Linderstrøm-Lang and Duspiva (1935) found that the hydrolysis of wool by the secretion of the clothes moth was due to a proteinase which could act on wool only after the latter had been in contact with a sulfhydryl compound also secreted by the digestive system of the moth. The air rapidly oxidized the natural activator, whose action could be duplicated with thioglycolic acid. (4) Certain activators may in reality be inhibitors of poisoners to the enzymes. This is suggested by the fact that, as some enzymes are progressively purified, less response is shown to the activator added; urease is an example of this (Sumner and Kirk, 1932).

Several classes of compounds are inhibitors, paralyzers, or poisoners of the enzymes. These compounds generally denature proteins and protoplasm. The salts of silver, gold, and mercury are markedly toxic in very low concentrations; copper, nickel, lead, zinc, and iron salts are less toxic, essentially in the order given. Salts of the alkaline and the alkaline earth metals are often used in making extracts and are not toxic except in concentrations above 2 per cent when they begin to act as precipitating agents. Oxidizing agents are generally harmful, as are the reagents for the amino group, such as nitrous acid and formaldehyde. Contact with ether and alcohol, except for short periods of time, should be avoided because these compounds denature proteins and hence inactivate enzymes.

Antienzymes are natural products which inhibit enzyme activity. Onslow (1917) has isolated from the skin of the underside of the rabbit, where the hair is white, a fraction which will inhibit the action *in vitro* of the tyrosinase isolated from that portion of the skin supporting dark hairs. Northrop (1939) crystallized from the pancreas a polypeptide which, under certain conditions, inactivates trypsin. Herriott (1941) isolated a crystalline antipepsin from solutions of pepsinogen. Harned and Nash (1932) separated an antitrypsin fraction from the roundworm of

hogs. Balls and Swenson (1934) obtained an antitrypsin from the thin portion of raw white of egg. It is also known that gastric mucin and the plant pectins exert an antipeptic function, probably by adsorbing part of the enzyme; accordingly these are used clinically for treating gastric ulcers.

PREPARATION AND PROPERTIES

An enzyme is seldom used in a pure or even in a comparatively pure form. The preparations range in composition from tissues containing relatively low concentrations of the enzyme to the pure crystalline product. Some commercial enzymatic preparations are merely ground tissues, and these may even contain inert diluents; for instance, the tanner's bate is made from ground pancreas diluted to the desired activity with wood flour. It is recalled that the zymase required in breadmaking is introduced in living yeast.

Many solid commercial products are prepared by first making an extract of the tissues with water, dilute acid, alkaline, saline, or glycerol solutions. At times preliminary freezing, grinding, drying, or the addition of toluene, acetone, or chloroform is resorted to in order to rupture the cells. The desired fraction is precipitated with alcohol or salted out with a highly soluble salt such as ammonium sulfate. It is to be noted that these procedures yield preparations rich in proteins, among which will be found the enzymes. This is essentially the degree of purification represented by such commercial preparations as malt and taka-amylase, papain, and trypsin.

Certain experimenters have further fractionated these preparations to obtain solid products of higher activity per unit of dried material. Dialysis, electrodialysis, selective adsorption, and a further fractionation from concomitant proteins are the usual procedures. The ultimate objective of such procedures is a preparation of constant activity per gram of dried material, and in several cases methods have been devised that yield the product in crystalline form of constant composition.

Care must be taken to exclude contaminating microorganisms from aqueous systems of enzymes and their substrates. Most antiseptic solutions function by denaturing proteins; loss of catalytic function parallels such denaturation of enzymes (Northrop, 1939). For this reason the use of most disinfectants should be avoided. Toluene has been employed in many cases to inhibit the growth of fungi and bacteria because it is comparatively harmless. A better procedure is to preserve the enzyme in the dry state until the time for its use or to store the aqueous suspension at low temperatures.

The composition of enzymes has received considerable study. Certain enzymes have been repeatedly crystallized until they are constant in

composition, physical properties, and activity. The first so obtained was urease (Sumner, 1926), a globulin with a molecular weight of 483,000. Pepsin, trypsin, chymotrypsin, and carboxypeptidase have been characterized as crystalline proteins. For each of these proteinases, the zymogen has also been obtained in the crystalline state. The literature on the preparation and properties of these proteinases is summarized by Northrop (1939). Among enzymes obtained as crystalline conjugated proteins are: catalase (Sumner and Dounce, 1937; also Laskowski and Sumner, 1941), which is an iron porphyrin protein; and the yellow enzyme of Warburg, a flavoprotein of which the prosthetic group is riboflavin pyrophosphate (Theorell, 1934). Crystalline tyrosinase is found to be a copper protein (Dalton and Nelson, 1938) and carbonic anhydrase is a zinc protein (Scott and Mendive, 1941). The nature of the linkage of the metal to the protein has not been established.

Other enzymes have been prepared in a high state of activity but have not been obtained in the crystalline form. Caldwell and Doebbeling (1935) obtained a very active amylase preparation which gave the usual qualitative tests for a protein and contained 16 per cent of nitrogen. Furthermore, the loss in enzymatic activity accompanied the denaturation of the protein. These facts have been interpreted to indicate that the enzyme is protein in nature. Many preparations of enzymes give the usual protein tests but contain a lower percentage of nitrogen than that generally encountered in proteins. These findings have led to the implied assumption that most, if not all enzymes, when completely purified and characterized, will be found to be protein in nature. It is to be noted that Lutz and Nelson (1934) have prepared a highly active yeast invertase which does not respond to any protein precipitation reagent.

The protein nature of enzymes confers upon them colloidal properties. Their molecular weights range from 20,000 for invertase, through values like 37,000 for pepsin and 248,000 for catalase, up to the value of 483,000 reported for urease. These have been calculated from rates of diffusion, from osmotic pressures, and from sedimentation velocities (Northrop, 1939; Sumner, Gralén, and Eriksson-Quensel, 1938). The enzymes diffuse very slowly and do not pass through a dialyzer; on the other hand, the coenzymes are of smaller size and can be removed by dialysis. Like other proteins, the enzymes exhibit an isoelectric point; on the acid side they are positively charged, and on the alkaline side they are negatively charged. The ionic properties make the enzymes particularly sensitive to changes in hydrogen-ion concentration, and their protein constitution renders them thermolabile. The enzymes often show increased activity in the presence of salts, perhaps due to their increased dispersion; this again is a property arising from the protein nature of most enzymes.

FACTORS INFLUENCING THE RATE OF REACTION

Whenever enzymes are employed it is necessary to give consideration to several factors that influence the rate of reaction. To determine the extent of these influences it is customary to conduct trial runs in which all conditions or factors are kept constant except the one being considered.

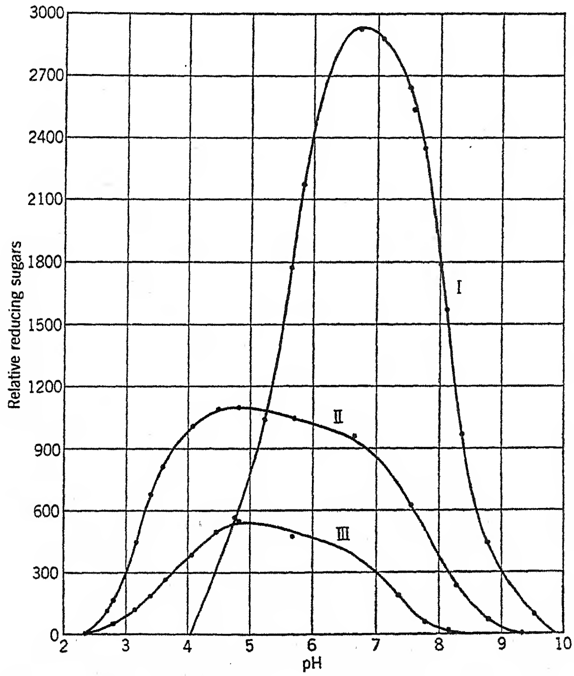

Fig. 1.—Influence of *p*H upon the activity of (I) pancreatic amylase, (II) malt amylase, (III) taka-amylase.

This has the limitation that the condition for the optimum effect of one factor may shift with a change in another variable factor. As an example, the optimum temperature often varies with the time duration of the run. It is therefore necessary either to determine the effects of several factors simultaneously in order to arrive at a set of conditions or factors which together approach the optimum state, or to recognize that the factor or factors determined separately give the optimum activity under the conditions empirically selected. The latter procedure is the one more often employed.

The influence of the hydrogen-ion concentration is of the greatest importance in enzyme studies; in fact, it was in this connection that Sørensen

(1909) first emphasized the significance of the hydrogen-ion activity. Its control is almost invariably attained by the use of buffered solutions. When the activity of an enzyme is plotted as a function of the hydrogen-ion concentration (*p*H) of the medium, the curve shows a point or a zone of optimum activity; this is illustrated in Figure 1 (Sherman, Thomas, and Baldwin, 1919).

Similar data are available for all enzymes that have been extensively studied. In general, the enzymes from fungi and plants are most active in the range of *p*H 4 to 6.5, and those of the glands and of the blood from the higher animals function best from *p*H 6.5 to 8. Pepsin is most active in a medium of *p*H 1.5 to 2, whereas arginase, bone phosphatase, and many of the oxidases give optima above *p*H 8. It is observed that enzymes from various sources may show different hydrogen-ion optima (*cf*. Fig. 1), which is not surprising, as they may be designed to operate in different media.

The nature of the substrate may influence the optimum value. Willstätter, Grassmann, and Ambros (1926) pointed out that papain showed its maximum activity at the isoelectric point of the protein substrate: the optimum was *p*H 5.0 for gelatin and albumin peptone, both of which have isoelectric points of 4.8; but it was *p*H 7.2 for fibrin, which has an isoelectric point of 7.2 also. Northrop (1922a) found that pepsin and trypsin had different optima and that these coincided with the regions in which the protein substrates existed largely in ionic form. Pepsin acts upon the cationic or positively charged form, and trypsin upon the protein anion; this is shown by the data of Table II. The isoelectric point of pepsin is at

TABLE II

OPTIMUM *p*H FOR PEPTIC AND TRYPTIC ACTION IN RELATION TO THE ISOELECTRIC POINT OF THE SUBSTRATE (Northrop, 1922a)

Protein	Isoelectric point of protein	*p*H for optimum action by	
		Pepsin	Trypsin
Casein	4.6	1.8	8.2
Hemoglobin	6.8	2.2	8.7
Gelatin	4.7	2.0	7.4

*p*H 2.7, whereas that of trypsin is at 7.0; consequently, pepsin is in the cationic form when it acts upon the cationic substrate, and trypsin is in the anionic form as it acts upon the substrate of the same charge. These facts rule out as an explanation the formation of a coacervate, that is, a phase separation of a complex composed of oppositely charged hydrophilic particles. The shift in the optimum hydrogen-ion concentration with different substrates is not necessarily confined to the proteases, for Sullivan and Howe (1933) observed a similar effect with wheat lipase.

In a few cases it has been observed that the quantity of substrate may alter the optimum. Table III illustrates a slight shift toward the acid region as more favorable with the higher concentration of urea. In most cases a variation in the concentration of the substrate produces only a change in the kinetics of the reaction (*vide infra*).

TABLE III

OPTIMUM *p*H FOR UREASE ACTION WITH VARIOUS BUFFERS AND WITH VARIED SUBSTRATE CONCENTRATION (Howell and Sumner, 1934)

Buffer	0.1% urea as substrate *optimum* pH	2.5% urea as substrate *optimum* pH
Acetate	6.7	6.4
Citrate	6.7	6.5
Phosphate	7.6	6.9

Table III shows that the composition of the buffer may alter the optimum hydrogen-ion concentration. The variation may be due in part to the different total ionic strengths employed; this is suggested by a study on malt amylase by Sherman, Caldwell, and Boynton (1930), who found that the optimum activity was at *p*H 4.5 to 4.8 in a 0.00005 *M* acetate buffer and increased to the range of 5.0 to 5.4 with a 0.1 *M* acetate buffer. Similarly, the optimum condition varied progressively from *p*H 4.5 to 4.9 with phosphate buffers ranging from 0.00005 to 0.1 *M*.

The presence of concomitant materials may shift the optimum hydrogen-ion concentration, possibly because of their effect of protecting the enzyme in a medium in which it is highly active but nevertheless is rapidly denatured. Willstätter, Haurowitz, and Memmen (1924) found that canine gastric juice gave the maximum lipase activity at *p*H 5.5 to 6.3. After electrodialysis, the zone moved to *p*H 6.3 to 7.1. When the dialyzed juice was subjected to adsorption upon kaolin and the enzyme leached off, the optimum activity was found at *p*H 7.1 to 7.9.

The time duration and the temperature of a run have marked effects upon the optimum hydrogen-ion concentration, which fact will be discussed later.

It is not generally known what phase of the reaction is influenced by a change in the acidity of the medium. The effect may be on the formation of an unstable complex of the enzyme and its substrate, on the rate of decomposition of this complex, on the stability of the enzyme, or on the substrate by production of some slight change such as a tautomeric shift. It can be shown for many enzymes that the downward slope of the activity–*p*H curve, on the alkaline side of the optimum hydrogen-ion concentration, parallels the denaturation curve of the enzyme in the same range. With

some enzymes the optimum activity is not at the point of greatest stability. Trypsin is most stable in the range from pH 5 to 6, whereas its optimum activity is always in a more alkaline range; similarly, pepsin is most stable at pH 4, but its activity is most pronounced at a lower level. By contrast, yeast invertase is most stable at its isoelectric point, where it also displays the greatest catalytic effect.

The temperature also has a marked effect upon the rate of enzyme activity. If the initial rates of enzyme activity are plotted as a function of temperature, a curve similar to that of Figure 2 is obtained. The quotient, $Q_{(t^\circ+10^\circ)/t^\circ}$, or Q_{10}, is obtained by dividing the amount of product yielded at one temperature into that yielded at a temperature 10° higher.

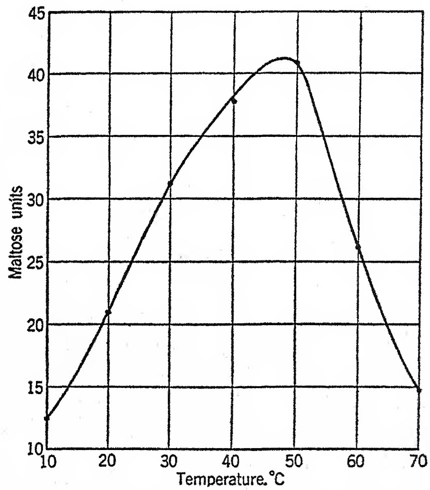

Fig. 2.—Effect of temperature upon the yield of maltose from starch by the action of malt diastase.

For most chemical reactions Q_{10} is in the neighborhood of 2; for most physical changes it is about 0.04. In the writer's laboratory the following values for Q_{10} were obtained with malt amylase: 10° to 20°, 1.67; 20° to 30°, 1.50; 30° to 40°, 1.12; 40° to 50°, 1.08; 50° to 60°, 0.62; 60° to 70°, 0.59. The stimulating effect of the increased temperature is offset by the denaturation of the enzyme by heat. Because the denaturation has a distinctly high temperature coefficient, the net result is reflected in progressively smaller increments of increase in activity with a rise in temperature until the point represented by the highest region of the curve is reached. At temperatures above this point, denaturation is of the greater importance. If it is reasonable to assume that the $Q_{20^\circ/10^\circ}$ would obtain throughout pro-

vided there was no denaturation, one would expect to obtain 7.7 times as much hydrolysis at 50° as at 10°, whereas the value obtained was 3.07, which is 40 per cent of the expected value. It is often assumed that 60 per cent of the enzyme is therefore destroyed at that point. Obviously this cannot be tested by maintaining the enzyme at 50° for a stated time before the substrate is added, because it is known that a much greater destruction results when the enzyme is not protected by the presence of the substrate. The enzymes resemble the proteins and the bacterial lysins in which the temperature coefficients of denaturation are the highest known among chemical reactions (Eyring and Stearn, 1939). Certain of the bacterial oxidases show a constant Q_{10} over a considerable range, presumably because they are unusually heat stable.

Based on these considerations, the following practical rules are now accepted. When an extract is raised from room temperature to the boiling point, all enzyme activity is destroyed. Often a temperature of 70° for 5 minutes will suffice to destroy enzyme action. Because the rate of inactivation of an enzyme is a function of time as well as of temperature, many aqueous enzyme preparations lose most of their activity when kept at room temperature for a day or two.

The duration of the experiment is also an important consideration. The amount of change produced by a constant quantity of enzyme is directly proportional to the time involved, provided that two conditions are fulfilled. First there must be sufficient substrate so that its concentration is not a limiting factor; and second, which is more important, the enzyme must not be measurably inactivated by its environment. This will be referred to later under a consideration of the kinetics of reactions.

It is futile to describe the optimum condition for either hydrogen-ion concentration or temperature without reference to the other and to the time duration of the run. Usually it will be observed that optimum yields are obtained in the longer time runs made at lower temperatures and at hydrogen-ion concentrations that are closer to neutrality. For blood catalase (Bodansky, 1919) the optimum is at pH 7.5 for a 10-minute run, but the enzyme acts best at pH 12 when the time is shortened to 1 minute. The beta-amylase of malt is most active at pH 4.3 at a temperature of 25°C, but as the temperature of the run is increased the optimum hydrogen-ion concentration shifts nearer to neutrality, until at 69° the maximum activity is at pH 6.0 (Olsen and Fine, 1924). Similarly, the optimum for tryptic hydrolysis of gelatin shifts from pH 10.5 to pH 7 as the temperature is raised from 28° to 55°C (Palitzsch and Walbum, 1912).

The concentration of the enzyme affects the reaction velocity. For short times and with a liberal excess of the substrate the velocity is generally proportional to the amount of enzyme employed. Under these re-

stricted conditions a constant amount of change will be produced if the time and the enzyme concentration are so varied that their product is a constant. This was shown for pepsin by Northrop (1920).

A few systems appear to obey the Schütz rule (1885) that the amount of change is proportional to the square root of the enzyme concentration. This was first announced for the digestion of egg albumin with crude pepsin. Northrop (1920) demonstrated that the free pepsin was the only catalyst and that the products of the reaction tend to combine with the enzyme; this gives rise to data that approach the Schütz rule. For such conditions he proposed a first order reaction (*vide infra*) in which the term $a/(a - x)$ is corrected by deducting the value of x. Northrop (1922) made a critical study of trypsin and found that the Schütz rule held over a range of time variations. Kuhn (1925) recalculated certain data on lipase and found that the Schütz rule held.

KINETICS OF ENZYME ACTION

Much information on the nature of enzyme action has been obtained from studies on the rate of the reactions catalyzed. For this purpose it is necessary to determine the amount of change produced over successive short time intervals. Van Slyke (1942) has presented a review discussion on the kinetics of the hydrolases.

The amount of change produced is often constant over several successive time periods. This can be represented by the equation $x = kt$, or $k = x/t$, where x is the amount of change measured at the various periods of time t, and k is the velocity constant for the system. A reaction conforming to this law is of zero order; when x is plotted as a function of t a straight line results. It is curious that the value of x does not drop off as the substrate is consumed; the constant rate of change is postulated to depend, not upon the total concentration of substrate, but upon the concentration of a complex of the enzyme and the substrate, and this complex remains constant in amount throughout the determination. This situation can occur only when the enzyme is not destroyed and when the substrate is in great excess. Evidence for the existence of such a complex will be presented later.

Under other conditions a reaction of the first order will be observed. In these reactions the amount of change produced in successive periods is a function of the substrate concentration. Since the latter is constantly being consumed, the value of x diminishes proportionally and a parabola results when x is plotted as a function of t. The equation for the velocity constant is $k = 1/t \ln a/(a - x)$, where a represents the initial concentration of the substrate. If k is found to be constant, the first-order law de-

scribes the course of the reaction. A test often employed is to plot $\ln (a - x)$ as a function of t; a linear relationship will be found. Many hydrolytic reactions belong to this order because the second reactant (water) is in such large excess that its concentration does not appreciably change. Second-order reactions, whose rates depend upon the concentrations of two reactants that are constantly changing, have never been found among those catalyzed by enzymes.

Enzyme systems may obey the zero-order law where large concentrations of substrates are involved and the first-order law when the substrate is present in limited quantities. This has been shown for urease (Van Slyke and Cullen, 1914) and for invertase (Michaelis and Menten, 1913). Such observations are made over a limited period of time in order that the substrate may not be depleted to the point at which the zero-order reaction gradually gives way to one of the first order. However, if one wishes to study the complete course of the reaction from a high initial concentration of the substrate to the point at which it is almost completely consumed, Van Slyke (1942) proposes the over-all equation:

$$t = \frac{1}{K_c} \ln \frac{a}{a - x} + \frac{x}{K_d}$$

where K_c is the velocity constant for the combination of enzyme and substrate, and K_d is the velocity constant for the production of x. Van Slyke designates this as a "two-phase reaction." It will be observed that the first term on the right is similar to that of the first-order, and the second term to that of a zero-order, expression. When x is sufficiently small so that x/K_d is negligible, the equation reduces to the first-order, or "die-away," form.

For some reactions the data do not under certain conditions yield constant values for K when calculated from the laws given above. Fruton, Irving, and Bergmann (1941) found that the values of K calculated for a first-order reaction were constant for a run made at 25° but that they changed progressively with time from 0.0023 to 0.0009 when the hydrolysis was conducted at 40°. This drop was taken as a measure of the degree of inactivation of the enzyme, an interpretation which was strengthened by the observation that the values of K decreased less in the presence of cysteine, which is known to protect the enzyme. This explanation might have been further tested by totally inactivating the enzyme with heat, adding a fresh lot of the enzyme, and again determining the rate of reaction and the velocity constants. If the values obtained are similar to those of the initial run, one may conclude that the resultants of the reaction are not interfering but rather that the enzyme is being gradually inactivated.

THE ENZYME-SUBSTRATE COMPLEX

Enzymes exhibit colloidal properties, so it is not surprising that they may form a complex with their substrate by adsorption or by an unstable chemical union. When the substrate is present in excess of the amount required to "saturate" the enzyme, the complex will remain constant in composition, and its decomposition will result in the constant rate of reaction (zero order) often encountered in spite of the fact that the substrate is constantly diminishing in concentration. Needless to state, it is difficult to isolate such a complex since by its very nature it must be quite unstable.

Haldane (1930) cites several lines of evidence that point to the existence of some such complex in many systems. It is often observed that an enzyme is more stable in the presence of its substrate, which suggests that the seat of its denaturation may be in the groups protected by a union with the substrate. At times, an enzyme may be selectively removed by adsorption from solution onto its substrate. Thus pepsin and steapsin are readily removed by an insoluble protein and fat, respectively. Invertase has been adsorbed on colloidal iron oxide, from which it can be removed by solutions of sucrose or of raffinose—but not by water or by solutions of lactose or fructose.

Stern (1936) demonstrated spectroscopically that a combination occurs between catalase and ethyl hydrogen peroxide. The purified enzyme was brown in solution and showed absorption bands at 650, 646–620, and at 610 mμ. Upon adding the substrate, the solution changed color through the green to a red, with bands at 576–564 and at 540–528 mμ. When the reaction had been completed, the solution of the enzyme returned to its original state.

Michaelis and Menten (1913) have derived a numerical expression (K_m) for the extent of such complex formation. It is the equilibrium constant for the reaction: enzyme + substrate $\rightleftharpoons$ complex. K_m is numerically equal to that concentration of the substrate which gives one-half the maximum initial velocity: $K_m = S(V/v - 1)$, in which S is the concentration of the substrate, V the maximum velocity attained by any substrate concentration, and v the velocity varying with S. Van Slyke and Cullen (1914) reached essentially the same equation by considerations predicated on the velocities of the reactions involved.

The values for K_m reported for the hydrolases range from 0.0005 M for an esterase to less than 5 per cent for a protease; most values are in the neighborhood of 0.01 M. The oxidizing enzymes possess lower values, in the region from 10^{-8} M to 0.001 M.

It has been shown by Michaelis and Menten (1913) that the K_m

value of a single invertase preparation did not vary with its concentration, but Kuhn (1923) found that values for preparations from various yeast sources differed somewhat; this is not surprising. Josephson (1924) reported that the K_m value was constant over a range from pH 4 to 8. These data point to the conclusion that K_m is essentially constant for a given system of enzyme and substrate.

NUMERICAL EVALUATION OF ENZYMES

In order to assay an enzyme preparation, it is necessary to determine the catalytic effect of that enzyme under specific conditions such that the amount of change produced can be translated into the quantity of enzyme required to effect that change. An arbitrary amount of change is designed as one activity unit. Thus Sumner and Hand (1928) define the urease activity unit as that amount of enzyme which will produce 1 mg of ammonia nitrogen from 30 mg of urea in 5 min at 20°C and at pH 7.0 in a phosphate buffer. It was found that, under the conditions selected, the amount of ammonia produced was a linear function of the amount of enzyme added and of the time duration. In other words, the reaction was of zero order.

Many assay methods are so designed that the course of the reaction gives a constant amount of change per unit time. This permits the enzyme to function at its maximum speed; consequently, the time duration need be only long enough to obtain an amount of change which can be accurately measured. In order to insure an environment in which the course of the reaction is of zero order, certain conditions must be observed, and these are determined empirically. The requirements common to all these methods are that the substrate must be present in sufficient concentration to insure saturating the enzyme throughout the run and that the enzyme be not inactivated under the conditions employed.

In some cases, conditions are employed under which the enzyme functions at a rate described by a reaction of the first order. Such a determination of activity is also made under prescribed conditions and the substrate is employed in a dilute solution. Fruton, Irving, and Bergmann (1941) made use of this method in their studies of peptidases. It is necessary to calculate the values of k for a first-order reaction and to relate these in some arbitrary way to activity units. Euler and Svanberg (1919) employed a similar method to calculate the activity of invertase.

A third method of measuring enzyme activity uses the inverse time–enzyme relationship, which states that the time required to decompose a given fraction of the substrate will vary inversely as the amount of enzyme present. This holds, whatever the kinetics of the reaction, but is

also limited to conditions under which the enzyme is not inactivated. Van Slyke and Cullen (1914) employed this method in part of their work on urease.

In these various methods the potency of the enzyme preparations are described in terms of units of their activity under prescribed conditions. It would be possible to describe the concentration of a crystalline enzyme in terms of micromolar concentration, but this would be of little value unless the equivalent activity units were determined.

REVERSIBILITY OF ENZYME ACTION

An ideal catalyst should be able to hasten the attainment of equilibrium when approached from either side, as has been demonstrated in many cases, both with inorganic catalysts and with enzymes. Several of the glucosides, esters, glycerides, and urea are among the compounds most readily synthesized with the aid of enzymes. In many cases, the synthetic reversals of hydrolytic reactions have been only partially successful *in vitro*. When concentrated mixtures of amino acids are subjected to protease action *in vitro*, complex "plasteins" are formed, which contain the peptide linkage but do not otherwise resemble the native proteins. Wasteneys and Borsook (1930) have surveyed the literature on the synthesis of proteins by enzymes.

Some of the rather unsatisfactory results may be due to a lack of knowledge as to the best conditions for synthesis. Sym (1936) obtained an 85 per cent yield of butyl benzoate after 48 hours at 37° with pancreatic lipase and bile salts in carbon tetrachloride. If instead of using the bile salts, he added water to the extent of only 4 per cent, the yield was reduced by 84 per cent.

It is possible that the hydrolytic enzyme of plants and animals may not be responsible for synthetic actions in all cases, even though they do effect some synthesis. Thus the amylases hydrolyze starch and glycogen, but the enzymatic synthesis of starch or of glycogen from glucose involves the intermediate formation of glucose-1-phosphate and phosphorylase; this subject is reviewed by Cori (1941). Similarly, urea can be synthesized *in vitro* from ammonium carbonate and carbamate (Mack and Villars, 1923), but its production in the higher animals is through the hydrolysis of arginine by arginase rather than through a synthesis with urease (Krebs and Henseleit, 1932).

BIBLIOGRAPHY

BALLS, A. K., and SWENSON, T. L. (1934). The antitrypsin of egg white. *J. Biol. Pres. Chem.* 106:409–419.

BODANSKY, M. (1919). A note on the determination of catalase in blood. *J. Biol. Chem.* 40:127–130.

BÜCHNER, E. (1897). Alkoholische Gärung ohne Hefezellen. *Ber.* 30:117–124.

CALDWELL, M. L., and DOEBBELING, S. E. (1935). A study of the concentration and properties of two amylases of barley malt. *J. Biol. Chem.* 110:739–747.

CHRZASZCZ, T., and JANICKI, J. (1936). Sulphuretted hydrogen as a factor in the determination of free and bound amylase in ungerminated cereals. *Biochem. J.* 30:342–344.

CORI, C. F. (1941). Phosphorylation of glycogen and glucose. In *Biological Symposia,* 5:131–140. Cattell Press, Lancaster, Pa.

DALTON, H. R., and NELSON, J. M. (1938). Crystalline copper-protein possessing tyrosinase activity. *J. Am. Chem. Soc.* 60:3085.

EULER, H. v., and SVANBERG, O. (1919). Saccharasegehalt und Saccharasebildung in der Hefe. *Z. physiol. Chem.* 106:201–248.

EYRING, H., and STEARN, A. E. (1939). The application of the theory of absolute reaction rates to proteins. *Chem. Revs.* 24:253–270.

FRUTON, J. S., IRVING, G. W., JR., and BERGMANN, M. (1941). On the proteolytic enzymes of animal tissues. The composite nature of beef spleen cathepsin. *J. Biol. Chem.* 138:249–262.

HALDANE, J. B. S. (1930). *Enzymes.* Longmans, Green, New York.

HARDEN, A., and YOUNG, W. J. (1906). The alcoholic ferment of yeast-juice. Part II. The co-ferment of yeast-juice. *Proc. Roy. Soc. London* B78:369–375.

HARNED, B. K., and NASH, T. P., JR. (1932). The protection of insulin by antiproteases, and its absorption from the intestines. *J. Biol. Chem.* 97:443–456.

HELFERICH, B., and SCHMITZ-HILLEBRECHT, E. (1935). Über Emulsin. XX. Der Einfluss von Neutralsalzen auf die Wirksamkeit von Mandelemulsin. *Z. physiol. Chem.* 234:54–62.

HERRIOTT, R. M. (1941). Isolation, crystallization, and properties of pepsin inhibitor. *J. Gen. Physiol.* 24: 325–338.

HOWELL, S. F., and SUMNER, J. B. (1934). The specific effects of buffers upon urease activity. *J. Biol. Chem.* 104:619–626.

JOSEPHSON, K. (1924). Über die affinität der Saccharase zu verschiedenen Zuckern. II. *Z. physiol. Chem.* 134:50–67.

KENDALL, J., and BOOGE, J. E. (1916). Studies on catalysis. I. The addition compounds of esters with organic acids. *J. Am. Chem. Soc.* 38:1712–1736.

KREBS, H. A., and HENSELEIT, K. (1932). Untersuchungen über die Harnstoffbildung im Tierkörper. *Z. physiol. Chem.* 210:33–66.

KUHN, R. (1923). Über Spezifität der Enzyme—II. Saccharase- und Raffinasewirkung des Invertins. *Z. physiol. Chem.* 125:28–92.

—— (1925). Physikalische Chemie und Kinetic. In C. Oppenheimer, *Die Fermente und ihre Wirkungen,* p. 240. Thieme, Leipzig.

KUNITZ, M. (1939). Kinetics of the formation of chymotrypsin from crystalline chymotrypsinogen and of trypsin from crystalline trypsinogen. *Enzymologia* 7:1–20.

LASKOWSKI, M., and SUMNER, J. B. (1941). Crystalline catalase from beef erythrocytes. *Science* 94:615.

LINDERSTRØM-LANG, K., and DUSPIVA, F. (1935). Beiträge zur enzymatischen Histochemie. XVI. Die Verdauung von Keratin durch die Larven der Kleidermotte. *Z. physiol. Chem.* 237:131–158.

LUTZ, J. G., and NELSON, J. M. (1934). Preparation of highly active yeast invertase. *J. Biol. Chem.* 107:169–177.

MACK, E., and VILLARS, D. S. (1923). Synthesis of urea with the enzyme urease. *J. Am. Chem. Soc.* 45:501–505.

MICHAELIS, L., and MENTEN, M. L. (1913). Die Kinetik der Invertinwirkung. *Biochem. Z.* 49:333–369.

NORTHROP, J. H. (1920). The effect of the concentration of enzyme on the rate of digestion of proteins by pepsin. *J. Gen. Physiol.* 2:471–498.

—— (1922). The inactivation of trypsin. II. The equilibrium between trypsin and the inhibiting substance formed by its action on protein. *J. Gen. Physiol.* 4: 245–260.

—— (1922a). The mechanism of the influence of acids and alkalies on the digestion of proteins by pepsin or trypsin. *J. Gen. Physiol.* 5:263–274.

—— (1939). *Crystalline Enzymes.* Columbia Univ. Press, New York.

OLSEN, A. G., and FINE, M. S. (1924). Influence of temperature on optimum hydrogen-ion concentration for the diastatic activity of malt. *Cereal Chem.* 1:215–221.

ONSLOW, H. (1917). A contribution to our knowledge of the chemistry of coat-colour in animals and of dominant and recessive whiteness. *Proc. Roy. Soc. London* B89:36–58.

PALITZSCH, S., and WALBUM, L. E. (1912). Über die optimale Wasserstoffionenkonzentration bei der tryptischen Gelatinverflüssigung. *Biochem. Z.* 47:1–35.

PRINGSHEIM, H., and BEISER, A. (1924). Über ein Komplement der Amylasen und das Grenzdextrin. *Biochem. Z.* 148:336–343.

PURR, A., and RUSSEL, M. (1934). Die Aktivierungsfähigkeit des Papains, angewendet auf eine Bestimmungsmethode physiologisch aktiver Stoffe im Blut. *Z. physiol. Chem.* 228:198–206.

SCHÜTZ, E. (1885). Eine Methode zur Bestimmung der relativen Pepsinmenge. *Z. physiol. Chem.* 9:577–590.

SCOTT, D. A., and MENDIVE, J. R. (1941). Chemical observations on carbonic anhydrase. *J. Biol. Chem.* 140:445–451.

SCOTT, E. M., and SANDSTROM, W. M. (1942). The activation of papain. *Arch. Biochem.* 1:103–109.

SHERMAN, H. C., CALDWELL, M. L., and ADAMS, M. (1928). A quantitative comparison of the influence of neutral salts on the activity of pancreatic amylase. *J. Am. Chem. Soc.* 50:2538–2543.

——, ——, and BOYNTON, H. H. (1930). A quantitative study of the influence of acetate and of phosphate on the activity of malt amylase. *J. Am. Chem. Soc.* 52:1669–1672.

——, THOMAS, A. W., and BALDWIN, M. E. (1919). Influence of hydrogen-ion concentration upon enzymic activity of three typical amylases. *J. Am. Chem. Soc.* 41:231–235.

SØRENSEN, S. P. L. (1909). Enzymstudien. II. Über die Messung und die Bedeutung der Wasserstoffionenkonzentration bei enzymatischen Prozessen. *Biochem. Z.* 21:131–304.

STERN, K. G. (1936). On the mechanism of enzyme action. A study of the decomposition of monoethyl hydrogen peroxide by catalase and of an intermediate enzyme–substrate complex. *J. Biol. Chem.* 114:473–494.

SULLIVAN, B., and HOWE, M. A. (1933). Lipases of wheat. I. *J. Am. Chem. Soc.* 55:320–324.

SUMNER, J. B. (1926). The isolation and crystallization of the enzyme urease. Preliminary paper. *J. Biol. Chem.* 69: 435–441.

—— and DOUNCE, A. L. (1937). Crystalline catalase. *J. Biol. Chem.* 121:417–424.

——, GRALÉN, N., and ERIKSSON-QUENSEL, I. (1938). The molecular weight of urease. *J. Biol. Chem.* 125:37–44.

—— and HAND, D. B. (1928). Crystalline urease. II. *J. Biol. Chem.* 76:149–162.

—— and KIRK, J. S. (1932). Is there a co-enzyme for urease? *Biochem. J.* 26:551–554.

SYM, E. A. (1936). Eine Methode der enzymatischen Esthersynthesen. *Enzymologia* 1:156–160.

THEORELL, H. (1934). Reindarstellung (Kristallisation) des gelben Atmungsfermentes und die reversible Spaltung desselben. *Biochem. Z.* 272:155–156.

VAN SLYKE, D. D. (1942). The kinetics of hydrolytic enzymes and their bearing on methods for measuring enzyme activity. In *Advances in Enzymology*, Vol. II, pp. 33–47. Interscience, New York.

—— and CULLEN, G. E. (1914). The mode of action of urease and of enzymes in general. *J. Biol. Chem.* 19:141–180.

WASTENEYS, H., and BORSOOK, H. (1930). The enzymic synthesis of proteins. *Physiol. Revs.* 10:110–145.

WILLSTÄTTER, R., GRASSMANN, W., and AMBROS, O. (1926). Substrat und Aktivitätsoptimum bei einigen proteolytischen Reaktionen. *Z. physiol. Chem.* 151:307–318.

—— HAUROWITZ, F., and MEMMEN, F. (1924). Zur Spezifität der Lipasen aus verschiedenen Organen. *Z. physiol. Chem.* 140:203–222.

CHAPTER II

AMYLASES

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Amylases, the enzymes which catalyze the hydrolysis of starches, are widely but very unevenly distributed in nature. They occur in different parts of higher plants, are formed by molds, yeasts, and bacteria, and are found in many tissues and secretions of the animal body. The most familiar examples are the amylases of the pancreatic juice, of the saliva (ptyalin), and of the cereal grains. In the earlier literature amylases were referred to as diastases and their action was known as diastatic action; at present, the terms amylase and amylase- or amylolytic-action are preferred.

The present chapter deals with the chemistry of amylases and of their action. For this reason, the discussion is not limited to amylases which are of particular importance in milling and baking but includes those from any source which appear to illustrate best our present information about the chemistry and mode of action of these important enzymes. The general properties of amylases and the methods used to measure their activities are summarized briefly. Some of these topics are then amplified and extended by more detailed studies of individual amylases or groups of amylases. No attempt has been made to give uniform discussions of the different amylases. With our present information, certain properties may be illustrated best with one amylase; others with another. Also, no attempt has been made to give a summary or catalogue of the miscellaneous information available about many important amylases. The topics considered are those which appear to extend our knowledge of the chemistry and mode of action of amylases or which give an indication of promising projects for future work. The applications of amylases in milling and baking technology are discussed in Chapter III.

Among the individual amylases, those of barley and of malted barley are discussed first and in some detail because they introduce the reader to

the two main types of amylases recognized at present, namely, the dextrinogenic, liquefying, or alpha-amylases and the saccharogenic or beta-amylases. The former are characterized by their ability to cause the rapid fragmentation of starches into reducing dextrins which give no color with iodine* (dextrinogenic action). These changes are usually accompanied by correspondingly rapid decreases in the viscosities of the starch pastes (liquefying action). In several cases, the products formed by dextrinogenic amylases have been found to exhibit falling, or alpha-mutarotation, hence the name alpha-amylases is also often applied to them. The saccharogenic amylases are characterized by their ability to split maltose rapidly from starches without markedly disrupting the rest of the starch molecule. The dextrins which remain after the action of saccharogenic amylases have relatively high molecular weights, are nonreducing, and retain the property of giving color with iodine. The maltose formed exhibits rising (dextrorotatory) or beta-mutarotation, hence the name beta-amylases. The terms dextrinogenic and saccharogenic are widely used at present as described above although neither is ideal. As will be explained more fully later, the dextrinogenic amylases exhibit so-called saccharogenic as well as dextrinogenic activities because they cause the formation of reducing substances; the saccharogenic amylases cause the formation of residual dextrins as well as of maltose.

GENERAL PROPERTIES OF AMYLASES

Chemical Nature. Those amylases which have been most carefully purified are proteins. They contain approximately 15 per cent nitrogen, give the usual protein color reactions, and yield amino acids upon hydrolysis (Osborne, 1895, 1896; Sherman, 1923; Sherman *et al.*, 1911, 1912, 1913a, 1915a, 1916b, 1926a,b, 1930a, 1931, 1934; Naylor *et al.*, 1925; Caldwell and Doebbeling, 1935; Ninomiya, 1940a; Werner, 1942). The evidence indicates that any treatment which denatures or disrupts the protein molecule causes a corresponding loss of amylase activity (Sherman, 1923; Sherman *et al.*, 1912, 1926b, 1930a, 1931, 1934; Caldwell and Doebbeling, 1935). So far, no evidence has been obtained for the presence in amylases of prosthetic groups such as are essential to the activities of oxidizing enzymes. Pancreatic amylase has been crystallized, and the recrystallized protein retained the amylase activity (Caldwell, Booher, and Sherman, 1931).

Reports that certain amylases are not protein have not been convincing for one or both of two reasons. Either the material found to be "protein-free" exerted no amylase activity and therefore was not an amyl-

* Unless otherwise stated, "colors with iodine" refer to those obtained by the use of dilute aqueous solutions of iodine and potassium iodide.

ase (Willstätter, Waldschmidt-Leitz, and Hesse, 1923; Sherman *et al.*, 1926a,b), or the enzyme solutions examined were too dilute to give positive protein color reactions even though they gave positive evidence of amylase activity (Waldschmidt-Leitz and Reichel, 1932a). Negative tests for proteins may be misleading because enzyme activity is usually measurable in solutions that are many times, often one thousand or more times, too dilute to give the usual protein color reactions (Sherman, 1923; Sherman *et al.*, 1926a,b, 1930a; Sumner, 1933; Northrop, 1934–35). Failure to obtain evidence of protein by the usual precipitation tests may sometimes be due to the presence of other substances which change its properties. Thus the presence of relatively high concentrations of carbohydrate appears to prevent the precipitation of invertase under certain conditions (Lutz and Nelson, 1934; Adams and Hudson, 1943). It has been stressed by many investigators that negative findings concerning the chemical nature of highly purified enzymes, vitamins, or hormones are unsatisfactory (Sherman, 1923; Sherman, Caldwell, and Adams, 1930a; Sumner, 1933; Northrop, 1934–35).

Activities and Stabilities. Amylases catalyze the hydrolysis of starches. Their action may be followed by measuring the disappearance of the substrate, or by measuring the appearance of its hydrolysis products. The latter may include maltose, reducing dextrins, nonreducing dextrins, other intermediate substances, and small amounts of glucose. The nature of the products formed, their relative proportions, and the order of their appearance in the reaction mixture depend upon the kind of amylase and the particular substrate used, as well as upon the conditions of the experiment.

Like other enzymes, amylases are relatively thermolabile; differences in this property are often employed to separate them. They also show extreme sensitivity to their chemical environment, which may have a favorable or an unfavorable influence upon their stabilities and activities. It is easy to inactivate an amylase irreversibly by chemical means. It is also possible to have a potentially active amylase which fails to give evidence of its activity because it is not provided with the proper environment—that is, with favorable kinds and concentrations of electrolytes and suitable hydrogen-ion activities. Purification procedures may remove electrolytes essential to the action of an amylase; and, unless these are again supplied, misleading results may be obtained. This is illustrated by the negligible activity of purified pancreatic amylase on dialyzed starch and its high activity when suitable anions are added in favorable concentrations (Kendall and Sherman, 1910).

No hard and fast statements regarding so-called optimal temperatures for amylase activities and stabilities can be made since these differ markedly with different amylases and with the same amylase under differ-

ent conditions. While the activities of amylases tend to increase with increasing temperatures, the inactivation of most amylases—probably the denaturation of their protein—also increases rapidly, especially as the region of rapid coagulation is approached. For this reason, measurements of amylase activities are usually made at a temperature considerably below that at which the rate of increase in the inactivation of the amylase equals the rate of increase in its activity. Temperatures between 20°C and 40°C are most frequently chosen for the measurement of amylase activities, but higher temperatures are desirable for many industrial processes.

Aqueous solutions of most amylases become progressively more thermolabile with purification and with dilution. As a rule, amylase solutions should be preserved at low temperatures and at high concentrations. Many of the impurities associated with crude amylases—proteins, amino acids, starches, other carbohydrates, and many electrolytes—tend to exert a stabilizing or protecting influence upon them. The proteins and starches may act as protective colloids or as buffers; the amino acids may act as buffers, and there is some evidence that they also protect certain amylases from hydrolysis (Sherman, 1923; Sherman *et al.*, 1912, 1921a,b, 1922a,b, 1923, 1925).

The hydrogen-ion activities of their solutions and substrates have a marked influence upon the stabilities and activities of amylases. For each amylase there is a range of hydrogen-ion activities over which it is most stable and most active, but the exact values depend upon a number of factors, among which the kind and concentration of electrolyte and the temperature and time of exposure are important. For this reason, statements of optimal hydrogen-ion activities should define the conditions under which they were established.

As would be expected, amylases, like other proteins, may be precipitated from their solutions by salts if the conditions are suitable. They readily form complexes with heavy metals and with other protein reagents. Losses of amylase activity may occur and may be reversible or irreversible depending upon the amylase, the agent, and the conditions.

In addition, like other typical proteins, amylases are often sensitive to the presence of relatively small concentrations of electrolytes. Whether this influence is favorable or unfavorable depends upon many interrelated factors, such as: the kinds and concentrations of other electrolytes present; the hydrogen-ion activities of the solutions; the temperature and time of exposure; and the kind, concentration, and degree of purification of the amylase and of its substrate. A few of the more carefully controlled studies will be reported for individual amylases; the interrelationships will be considered in some detail in connection with pancreatic amylase.

There are many indications that certain amylases are more or less

sensitive to light, depending upon its wave length (Waksman and Davison, 1926; Haldane, 1930; Laurens, 1933).

Natural sources of enzymes such as grains and yeasts have been reported to contain specific activators and inhibitors of amylase activities. The evidence for such substances is impossible to evaluate when the interrelated factors which influence amylase activity have been poorly controlled or incompletely reported, or when crude extracts have been used. Certainly such specific activators are not essential to the attainment of a high degree of activity with a number of highly purified amylases. Caldwell and Doebbeling (1935) have shown that highly purified preparations of the alpha- or the beta-amylase of malted barley exert exceedingly high activities in the presence of purified starch at favorable hydrogen-ion activities, although no source of natural activators is added (see also Weidenhagen, 1933).

The *Amylokinase* found by Waldschmidt-Leitz *et al.* (1931, 1932a,b) in malted barley is typical of the activators which may occur in natural materials. Kneen and Sandstedt (1943a) have recently reported the separation from wheat of an inhibitor of the activity of certain animal and bacterial amylases.

Measurement of Activities. The dextrinogenic activities of amylases may be followed quantitatively in a number of ways. These include measurements of the changes in the color (Roberts, 1881; Wohlgemuth, 1908; Sherman and Thomas, 1915b; Samec, 1935; Blom, Bak, and Braae, 1936; Hanes and Cattle, 1938; Sandstedt, Kneen, and Blish, 1939) or in the e.m.f. value of the reaction mixtures when starches and their hydrolysis products are treated with iodine (Bates, French, and Rundle, 1943; Caldwell, unpublished); measurements of changes in the turbidity (Rona and van Eweyk, 1924); measurements of the decrease in the material precipitated by alcohol (Caldwell and Hildebrand, 1935); and measurements of the decrease in the viscosity of reaction mixtures (Józsa and Gore, 1930; Józsa and Johnston, 1935; Lüers and Löther, 1935; Waldschmidt-Leitz and Mayer, 1935; Blom and Bak, 1938).

The first type of measurement mentioned above is the basis for the classical Wohlgemuth (1908) method, which—with certain modifications—is still widely and successfully used. The essential steps are given here: A series of tubes, containing—in the same total volume—the same concentration of a buffered starch paste but increasing concentrations of the amylase, is held at the desired reaction temperature for a suitable period of time and treated with a given volume of dilute iodine solution. With a suitable amylase solution acting on a properly prepared substrate, a series of colors ranging from blue through violet, red, orange, and yellow, to "colorless" is obtained. The colors are compared with a standard. The weight

of starch hydrolyzed to a selected end-point by a given weight of amylase may be calculated with considerable precision (Sherman and Thomas, 1915b; Sandstedt, Kneen, and Blish, 1939).

Many investigators have chosen as the end-point the "achromic stage" at which no color is given with iodine. Greater precision is obtained with an end-point that indicates an earlier stage of hydrolysis at which small differences in the concentration of the amylase produce marked changes in color. Wohlgemuth (1908) himself and many subsequent investigators prefer the first clear red color where no trace of the blue is discernible. Sandstedt, Kneen, and Blish (1939) suggest as the standard a commercial dextrin which gives a red-brown color with iodine. Spectrophotometric measurements of the colors with iodine have also been used (Hanes and Cattle, 1938).

One of the characteristic properties of certain amylases is their liquefying action or their ability to cause rapid decrease in the viscosities of starch pastes. This property is extremely important in nature and in many industrial processes. It has formed the basis for numerous methods designed to measure the activities of amylases and to distinguish between them. Many difficulties are encountered in the quantitative application of such measurements because they are influenced by so many factors. The results obtained depend to an unusual extent upon the methods and conditions used in the preparation of the substrate and for the viscosity measurements (Chrzaszcz and Janicki, 1932). Among the factors which influence the viscosity of an aqueous starch paste are the method and temperature of making the paste, the kind and concentration of electrolytes, the hydrogen-ion activities, and the method and conditions under which the measurements are made. Even when all these factors have been carefully controlled, the aqueous pastes obtained from whole starches are unhomogeneous (Meyer, 1942), and the average values which they yield in viscosity measurements are not sufficiently reproducible to give a suitable base line for the comparable and quantitative evaluation of the liquefying action of amylases. This difficulty has led to the use of special substrates prepared from starches made soluble by chemical or mechanical means (Lintner, 1886; Józsa and Gore, 1930; Józsa and Johnston, 1935; Waldschmidt-Leitz and Mayer, 1935; Blom and Bak, 1938). When employed with due regard to the factors mentioned above, these soluble starches permit satisfactorily reproducible measurements of the liquefying action of an amylase (Dickson, 1943), and their use has resulted in much valuable information about amylases. On the other hand, solubilized starches are highly modified and therefore are not ideal substrates for the study of the way in which amylases act. This handicap is especially important in the study of the liquefying action of amylases, which, perhaps more than any other evidence

of amylase activity, is concerned with the very early changes in the substrate. As soluble starch has already lost much of its original viscosity, it is evident that linkages which might normally be attacked by an amylase may already have been altered or ruptured before the addition of the enzyme. It is also evident that the changes in the substrate may influence the action of one amylase more than that of another. Such considerations throw doubt on the significance of quantitative comparisons of the liquefying action of amylases from different sources when the measurements are based on the use of soluble starches. It is to be hoped that the new work with starches and their components will soon afford more satisfactory substrates for the study of this important property of amylases.

Although maltose is not the sole, and in some cases not even an important, reducing product formed from starches by amylases, it has become customary to convert the reducing values of amylase reaction mixtures to their equivalent of maltose and to report the so-called saccharogenic activities of amylases in terms of maltose or in terms of the percentage yield of this sugar which could be obtained theoretically from the substrate. This convention is satisfactory for comparative purposes provided its limitations are fully appreciated. Because of this convention, dextrinogenic amylases are endowed with so-called saccharogenic activities even when the reducing values of their reaction mixtures are due largely to reducing dextrins or to reducing sugars of higher molecular weights than maltose.

The saccharogenic activity of an amylase can be measured by any method which will permit the quantitative determination of maltose or of glucose. The methods most commonly used are based upon the reduction of cupric or of ferricyanide ions, or upon the reduction of iodine. The iodine-reduction methods are stoichiometric while those based upon the reduction of cupric or of ferricyanide ions are not. Therefore, for an unknown mixture of reducing substances, more information may be obtained by use of both types of method than by either one alone. Agreement between the two indicates the presence of maltose alone; discrepancies are evidence of the presence of other reducing substances. Correlation of changes in reducing value with changes in optical rotation also gives considerable information about the products formed.

Regardless of the kind of amylase activity under investigation it is essential that the measurements of enzyme activity yield values which are directly proportional to the concentration of amylase. In order to obtain such measurements two types of procedures have been used. The first depends upon the hydrolysis of a constant fraction of the substrate. Measurements may be made either of the time required to produce this constant change by a unit weight of enzyme or of the amount of enzyme necessary to produce this change in a constant time. The time required to produce a

definite change in the substrate is inversely proportional to the concentration of enzyme, and the results of these measurements are usually expressed in terms of the amount of substrate hydrolyzed to a selected end-point by unit weight of enzyme in a definite period of time. This type of procedure has been used most frequently for measurements of the dextrinogenic activity of an amylase (Roberts, 1881; Wohlgemuth, 1908; Sherman and Thomas, 1915; Sandstedt, Kneen, and Blish, 1939) but may also be used for measurements of the saccharogenic activity (Lintner, 1886; Ballou and Luck, 1940, 1941).

In the second type of procedure, the extent of the hydrolysis of the substrate varies. The method of evaluating the activity of an amylase by this procedure depends upon the particular kind of amylase under investigation; many methods of calculation have been developed which relate the extent of hydrolysis of the substrate to the activity of the amylase. For beta-amylase (free from alpha-amylase) from barley or wheat, Kneen and Sandstedt (1941) have demonstrated a linear relationship between the extent of hydrolysis and the concentration of the amylase up to 30 per cent starch conversion, and for unmodified malt extract (alpha- and beta-amylases) up to 40 per cent starch conversion. No such simple linear relationship has been observed for the saccharogenic activities of alpha-amylases. Various methods of calculation utilizing the monomolecular reaction constants have been proposed that yield values directly proportional to the activity of the enzyme provided the measurements are made in the relatively early stages of hydrolysis (Kjeldahl, 1879–80; Sherman, Kendall, and Clark, 1910; Euler and Svanberg, 1920–21; Willstätter, Waldschmidt-Leitz, and Hesse, 1923; Hanes, 1932).

This second type of procedure has been used chiefly for the measurement of the saccharogenic activity of amylases, although it has also been used for evaluating the liquefying action of alpha-amylases by means of empirical relationships which have been established between the extent of liquefaction of the substrate and the concentration of the amylase (Józsa and Gore, 1930; Józsa and Johnston, 1935).

As the result of many investigations of the kinetics of the reactions catalyzed by amylases many units have been proposed to express amylase activities (Lintner, 1886; Sherman, Kendall, and Clark, 1910; Euler and Svanberg, 1920–21; Willstätter, Waldschmidt-Leitz, and Hesse, 1923; Józsa and Gore, 1930; Józsa and Johnston, 1935; Blom, Bak, and Braae, 1937; Sandstedt, Kneen, and Blish, 1939; Kneen and Sandstedt, 1941b). These units apply only to the particular enzyme for which they have been developed and under the conditions specified. For any one amylase it is possible to convert one unit to another provided empirical factors for such a conversion have been established by means of comparisons made under

the conditions defined for each unit. However, difficulties in the techniques involved in some of the methods as well as the lack of suitable standard substrates often make such comparisons difficult even when carried out by skilled operators (Anderson and Sallans, 1937; Dickson, 1943).

While comparisons of the activities of amylases from different sources are of great practical importance, their theoretical significance is questionable because of the heterogeneous nature of the substrates so far available and the variety of the products formed. It is to be hoped that the recent developments in our knowledge of the chemistry of starches will lead to universally acceptable substrates for the evaluation of amylase activities. The substrates chosen may be different for different kinds of amylase activities. The conditions will necessarily differ for different amylases.

Substrates. Most extractable amylases have relatively little or relatively slow action on sound raw starches. Therefore, starch substrates for amylase action are generally prepared in the form of pastes or solutions. Such substrates, when obtained by heating aqueous suspensions of whole starches, are heterogeneous and unsatisfactory for quantitatively reproducible measurements of amylase activities. For this reason it has become customary to use starches made soluble by chemical or mechanical means. The most widely used substrates for measurements of amylase activities are starches made soluble by acid treatment according to Lintner (1886) or by some modification of this method (Small, 1919). As already pointed out, starches made soluble by mechanical treatment have been especially recommended for viscosity measurements (Józsa and Gore, 1930; Józsa and Johnston, 1935; Waldschmidt-Leitz and Mayer, 1935; Blom and Bak, 1938).

The heterogeneous nature of starches has long been recognized. Samec (Samec and Blinc, 1938, 1939; Samec, 1940) has reviewed the literature dealing with their fractionation and structure. Meyer (1942) has summarized the evidence for two distinct types of starch components and has proposed a redefinition of the terms "amylose" and "amylopectin." He uses these terms for the straight-chain and for the branched-chain components of starches respectively. These terms will be used here (see also Meyer *et al.*, 1940c, 1941d; Meyer, 1943; Freudenberg *et al.*, 1938a, 1939, 1940a,b; Bates, French, and Rundle, 1943; Rundle and Baldwin, 1943).

The accumulated evidence of many investigators (summarized by Meyer, 1942) indicates that the straight-chain components or amyloses consist of unbranched chains of glucose residues in which the glucose units are united by α-1,4-glucosidic linkages (see Fig. 1). According to Meyer (1942), amyloses consist of 100 to 700 glucose residues. The amylopectins, or the branched-chain components of starches, consist of large branched molecules made up of glucose residues, most of which are joined by α-1,4-

glucosidic linkages but with branches formed by α-1,6-glucosidic linkages. Meyer (1942) concludes that amylopectins are made up of 500 to 2000 or more glucose residues. He believes that the evidence indicates that branch-

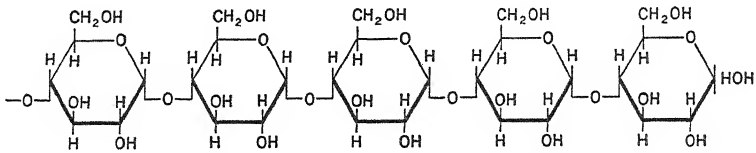

Fig. 1.—Section of an amylose chain.

ing occurs, on the average, at every twenty-fifth glucose unit and that "the outside branches of amylopectin consist of 15 to 18 glucose units, whereas the inside parts of the chains, between branch positions, are about 8 to 9

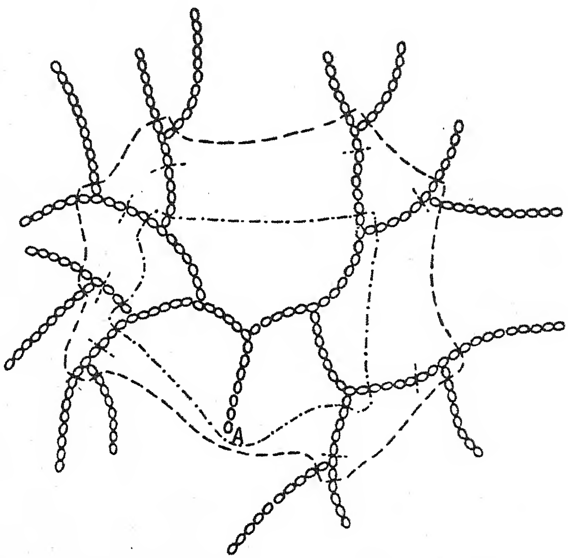

Fig. 2.—Schematic structure of amylopectin, showing successive stages in its enzymatic hydrolysis. A, aldehydic end-group; o, glucose unit.

— — — End of initial degradation by beta-amylase, yielding residual dextrin I.

- - - - - Hydrolysis continued by alpha-glucosidase, giving dextrin II, hydrolyzable by beta-amylase.

–·–·– End of further attack by beta-amylase, yielding residual dextrin III. (Meyer, 1942)

units long." A diagrammatic representation of amylopectin suggested by Meyer (1942) is given in Figure 2.

The evidence indicates that Meyer's conclusions regarding the

average structure of amylopectins are essentially correct, but many questions remain to be answered and investigators are continuing to study both the number of glucose residues which amyloses and amylopectins contain and the number and the positions of the branches in the amylopectin molecules. There is also uncertainty as to the number of amyloses and amylopectins which may be present in any given type of starch. Fox (1943) has reported that, in addition to the straight-chain component, cornstarch also contains two branched-chain components of widely different molecular weights. The evidence indicates that both of these amylopectins exist in the original cornstarch and are not broken down from a larger amylopectin, but this is not yet fully established (1943).

Recent investigations have introduced more satisfactory methods for the quantitative separation and study of amyloses and amylopectins (Bates, French, and Rundle, 1943; Bear and French, 1941; Beckmann and Landis, 1939a,b; Beckmann, unpublished; Meyer *et al.*, 1940a,d; Meyer, 1942; Rundle and Baldwin, 1943; Rundle and French, 1943; Schoch, 1942; Wilson, Schoch, and Hudson, 1943). The availability of more homogeneous and more definitely characterized substrates offers promising possibilities for advancing our understanding of amylase action.

Glycogens are the reserve carbohydrates of the animal kingdom. They form aqueous solutions which are characteristically opalescent and give a red-brown to red-violet color with iodine. Like starches, glycogens are hydrolyzed by amylases (Barbour, 1929; van Klinkenberg, 1932b; Glock, 1936; Šamec and Blinc, 1939; Meyer *et al.*, 1941a,b,c,d; Meyer, 1942, 1943); they yield glucose upon acid hydrolysis.

The evidence indicates that glycogens consist of large very highly branched molecules of glucose residues united by α-1,4- and by α-1,6-glucosidic linkages (Barker, Hirst, and Young, 1941; Meyer, 1942, 1943; Bridgman, 1942).

THE AMYLASES OF BARLEY AND OF MALTED BARLEY

The amylases of barley and of malted barley have been intensively studied by many investigators and offer a convenient basis for the discussion of other amylases. Outstanding among the early developments are the classical investigations of O'Sullivan (1876, 1879) which gave detailed information regarding the products formed when extracts of malted barley reacted with starch. His results and those of other investigators working with extracts of malted barley, prepared and used under different conditions, suggested the presence of more than one amylase (Grützner, 1876; Märker, 1878; Brown and Heron, 1879; Bourquelot, 1887; Lintner,

1886, 1887; Lintner and Eckhardt, 1890; Brown and Morris, 1890; Wijsman, 1890; Euler, 1922). This suggestion has been abundantly confirmed and extended by numerous workers.

Ohlsson (1922, 1926, 1930) developed methods for the separation of two of these amylases, the dextrinogenic or alpha-amylase and the saccharogenic or beta-amylase.

The separation from barley of a third type of enzyme which is particularly potent in starch-liquefying activity was reported by Waldschmidt-Leitz and Mayer (1935). They called it amylophosphatase because they found that it hydrolyzed phosphoric acid rapidly from starches (see also Mayer and Klinga-Mayer, 1940).

The presence of an enzyme, or enzymes, which causes the hydrolysis of raw starches has been reported in malted barley by a number of investigators (Brown and Heron, 1879; Brown and Morris, 1890). This action does not appear to be due to the beta-amylase (Blish, Sandstedt, and Kneen, 1938) and is currently associated with alpha-amylase activity (Stamberg and Bailey, 1939; Kneen, Beckord, and Sandstedt, 1941a; Burkert and Dickson, 1941; Kneen, 1943).

The Saccharogenic Amylase or Beta-Amylase

Action on Starches. The beta-amylase of barley and of malted barley causes the rapid formation of maltose from starches and from certain dextrins, and liberates this sugar as beta-maltose which shows rising or dextrorotatory mutarotation (Brown and Heron, 1879; Brown and Morris, 1890; Kuhn, 1925; Ohlsson, 1930; Freeman and Hopkins, 1936c). Moreover, there is abundant evidence that maltose is the sole significant reducing product and the sole significant product of low molecular weight formed from starches by beta-amylase. A brief summary of the evidence for the above statements follows.

The reducing values of the hydrolysis mixtures of beta-amylase correspond to the same yields of maltose whether they are determined by iodine-reduction or by copper-reduction methods (Blom, Bak, and Braae, 1936).

Osmotic-pressure measurements (Ohlsson, 1926) of the reaction mixtures of beta-amylase on soluble potato starch give initial values which are much higher than those given under similar conditions by the original substrate. At equilibrium, however, the osmotic-pressure values of the reaction mixtures correspond very closely to those given by the original substrate. These results indicate that relatively large numbers of readily diffusible molecules are formed from the starch without increasing appreciably the number of nondiffusible or slowly diffusible particles. The diffusible material agrees with maltose in its reducing value and in its optical rota-

tion. Taken altogether, these results indicate that maltose is formed and that it is liberated from the ends of the glucosidic chains without appreciably disrupting the rest of the molecule (Ohlsson, 1926).

When the reaction mixtures, taken at different stages of the action of beta-amylase on starch, are fractionated with alcohol, the alcohol-soluble or sugar fractions give reducing values which account for practically all of the reducing values of the reaction mixtures from which they come. These alcohol-soluble fractions also show good agreement with pure maltose in their reducing values, optical rotation, and rates of fermentation. Pure crystalline maltose has been obtained in high yields from these alcohol-soluble fractions (Baker, 1902; Blom, Bak, and Braae, 1936; Freeman and Hopkins, 1936a; Hanes, 1935b; Hopkins and Roberts, 1935; Syniewski, 1902, 1925).

An indication that traces of glucose may appear after the prolonged action of beta-amylase on soluble starch was reported by Hanes (1935b, 1937). These traces may have been due to cleavage of glucose from some intermediate hydrolysis product by a contaminating enzyme or may indicate merely that glucose remains after all possible maltose has been split from a glucosidic chain with an uneven number of glucose units. It should be noted, however, that Myrbäck (1943) reports that beta-amylase does not hydrolyze maltotriose made up of three glucose residues although it does hydrolyze the compound made up of four such residues (Myrbäck, Örtenblad, and Ahlborg, 1940c).

The limits in the hydrolysis of starches by beta-amylase have been extensively studied. The data given in Figure 3 were obtained by van Klinkenberg (1932b) when different concentrations of beta-amylase from barley acted on soluble potato starch at 40°C. They are typical of the action of this amylase. When sufficient enzyme is used, the reducing values of the reaction mixtures rise rapidly until 40 to 50 per cent of the theoretical yield of maltose has been reached and then more slowly to approximately 64 per cent. The rate of liberation of maltose during the early stages of the reaction depends upon the concentration of the beta-amylase, but the extent of the hydrolysis does not. Thus a limit is reached at approximately 64 per cent of the theoretical maltose—in spite of wide variations in the concentration of beta-amylase, provided the amylase is not contaminated with another enzyme.

The extent of the hydrolysis of starch by beta-amylase is not greatly influenced by relatively wide differences in the concentration of the substrate or in the conditions of the reaction (Baker, 1902; Ling and Nanji, 1925; Syniewski, 1925; Hopkins, Cope, and Green, 1933; Hanes, 1935b; Samec, 1935; Blom, Bak, and Braae, 1936; Tychowski, 1937; Samec and Blinc, 1939). Moreover, starches from a number of

different sources such as potato, wheat, buckwheat, barley, maize, rye, rice, apple, and arrowroot appear to be hydrolyzed to approximately the same extent by beta-amylase (Baker, 1902; Ling and Nanji, 1925; van Klinkenberg, 1932a; Hanes, 1935b, 1936, 1937; Tychowski, 1937).

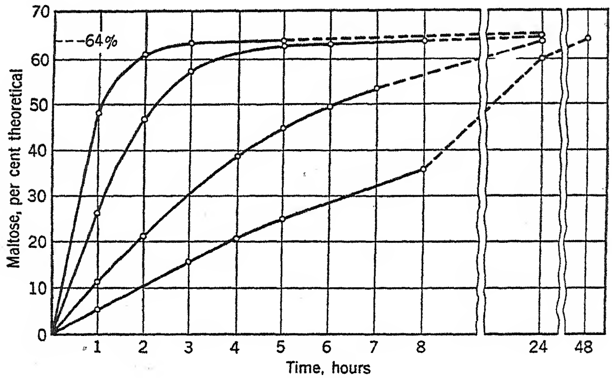

Fig. 3.—Influence of enzyme concentration on the action of beta-amylase from barley. Soluble potato starch, 1 per cent; pH, 5.0; 0.025 *N* citrate; 40° C; purified beta-amylase in relative concentrations of x, $2x$, $5x$, and $10\,x$ (van Klinkenberg, 1932b).

When the limit of the hydrolysis of starch by beta-amylase has been reached, the reaction mixture retains the property of giving a blue-violet or violet color with iodine. The color produced will depend somewhat upon the conditions of the action and the kind and concentration of the starch substrate; but, if the beta-amylase is not contaminated with other enzymes, the reaction mixture will show no tendency to change to the red or achromic stages even after prolonged amylase action (Hanes, 1935b; Myrbäck, 1936; Tychowski, 1937; Meyer, Wertheim, and Bernfeld, 1941d; Caldwell, unpublished).

The dextrins formed from starches by the action of beta-amylase have been separated at different stages of the reaction by precipitation with alcohol. They show progressive changes in properties as is indicated by the decreasing intensities of their colors with iodine (Blom, Bak, and Braae, 1936; Samec, 1935), by their decreasing optical rotation (Brown and Heron, 1879; Baker, 1902; Ling and Nanji, 1923), and by the evidence of their decreasing molecular weights (Ling and Nanji, 1923; Myrbäck, 1938a; Beckmann and Landis, 1939a,b). The dextrins, like the original starches, have negligible reducing values.

When the limit of the hydrolysis is reached, a characteristic resistant dextrin is left which retains many starchlike properties. This dextrin was recognized by early investigators. It was called erythrogranulose by

Wijsman (1889) and α-amylodextrin by Baker (1902). At the present time, the term residual dextrin is being widely used. This residual dextrin is practically nonreducing (Baker, 1902; Ling and Nanji, 1923; van Klinkenberg, 1932a,b; Samec, 1935; Beckmann and Landis, 1939b), appears to have a relatively high molecular weight (Baker, 1902; van Klinkenberg, 1932a,b; Samec, 1935; Beckmann and Landis, 1939b; Coles, 1941), and gives a blue-violet or violet color with iodine. It retains most if not all of the phosphoric acid present in the original starch (Stamberg and Bailey, 1939; Myrbäck and Kihlberg, 1943).

Action on the Components of Starches. Under suitable conditions, the amylose component of starches is completely hydrolyzed by beta-amylase (Meyer *et al.*, 1940b; Meyer, 1942; Hassid and McCready, 1943; Beckmann, unpublished; Caldwell, unpublished). Numerous earlier investigators have also reported the complete hydrolysis by beta-amylase of certain starch fractions which were similar to the amyloses discussed here. Failure to obtain complete hydrolysis of so-called amyloses has also been reported. Such failure may have been due either to contamination of the amylose with amylopectin or to retrogradation (the precipitation of the amylose from its solution) which occurs readily with amyloses. It is obvious that unless retrogradation is prevented, 100 per cent hydrolysis of even a pure amylose cannot be expected.

Meyer *et al.* (1940a) reported the partial (54.0 per cent) hydrolysis of a branched-chain component of cornstarch by beta-amylase and found this enzyme did not hydrolyze the α-1,6-glucosidic linkages. Failure to hydrolyze the α-1,6-glucosidic linkages was evident from the fact that the number of end-groups determined in the residual dextrin after the amylase action was the same as that of the original amylopectin. Myrbäck (1943) and Myrbäck *et al.* (1940a) also report that beta-amylase does not hydrolyze the α-1,6-glucosidic linkages of amylopectin. Hassid and McCready (1943) report 54 per cent hydrolysis by beta-amylase of an analyzed amylopectin component from potato starch. A sample of Lintner's soluble potato starch was analyzed (Caldwell, unpublished) by the method of Bates, French, and Rundle (1943) and found to contain approximately 22 per cent amylose and 78 per cent amylopectin. The yield of 64 per cent of the theoretical maltose from this starch by the action of beta-amylase agrees with that calculated for the complete hydrolysis of the amylose and the partial (54 per cent) hydrolysis of the amylopectin.

Mode of Action. While the study of starch structure by recently improved methods is far from complete, it offers an explanation for the mode of action of beta-amylase and for the formation of the resistant dextrin.

Beta-amylase causes the hydrolysis of maltose from the ends of glucosidic chains by the cleavage of α-1,4-glucosidic linkages. It is prob-

able that the maltose is hydrolyzed from the nonaldehydic ends of the glucose chains and that the aldehyde groups of the substrate remain unchanged. This conclusion is supported by several kinds of evidence. The dextrins formed by the action of beta-amylase resemble the starch from which they come in having negligible reducing value. Aldehyde groups do not appear to be essential to the action of beta-amylase as it causes the hydrolysis of dextrins after their aldehyde groups have been oxidized to the corresponding acids (Brown and Millar, 1899; Myrbäck, 1936; Örtenblad and Myrbäck, 1941b; Meyer, Bernfeld, and Press, 1940b). Beta-amylase causes the partial hydrolysis of amylopectins by the liberation of maltose from the nonaldehydic ends of the branches; the aldehydic ends are utilized to form the α-1,6-glucosidic linkages of the molecule (see Fig. 2). As pointed out above, other evidence indicates that beta-amylase does not cause the cleavage of α-1,6-glucosidic linkages or perhaps of the α-1,4-glucosidic linkages adjacent to and influenced by the latter (Meyer *et al.*, 1940b; Myrbäck, 1943). To summarize, beta-amylase appears to hydrolyze the amylose components of starches completely to maltose; to hydrolyze the amylopectin components only partially by the cleavage of maltose from the ends of the free branches of the molecule until the α-1,6-glucosidic linkages are approached or reached. The unhydrolyzed residue from the amylopectin forms the residual dextrin (see Fig. 2).

Chemical Nature. Purified preparations of beta-amylase have been obtained from barley and from malted barley by a number of workers (Sjöberg and Eriksson, 1924; Lüers and Sellner, 1925; Ohlsson, 1930; van Klinkenberg, 1932a). The most highly active are protein and give no evidence of a prosthetic group or for the need of a specific activator (Caldwell and Doebbeling, 1935; Caldwell, unpublished).

Conditions Which Favor the Activity. The data given in Figure 4 illustrate the influence of the hydrogen-ion activity of the substrate upon the activity of a highly purified preparation of malt beta-amylase (Caldwell and Doebbeling, 1935). Somewhat different hydrogen-ion activities have been reported for the optimal activity of beta-amylase when it acts under other conditions (Ohlsson, 1926; Andrews and Bailey, 1934; Hanes, 1935b; van Klinkenberg, 1932a, 1934). These differences illustrate a point already emphasized, that the conditions which favor the action of amylases are all interrelated and that, if one condition is changed, others also may change.

Anions such as chloride, nitrate, acetate, sulfate, and phosphate have no appreciable influence upon the activity of highly purified beta-amylase from malted barley provided the hydrogen-ion activities of the reaction mixtures are suitably controlled (Sherman *et al.*, 1930b,c; Caldwell and Doebbeling, 1935). Kneen *et al.* (1943b) report an unfavor-

able influence of calcium ions upon the stability of beta-amylase in extracts of malted barley especially when they are exposed to high temperatures or to high hydrogen-ion activities.

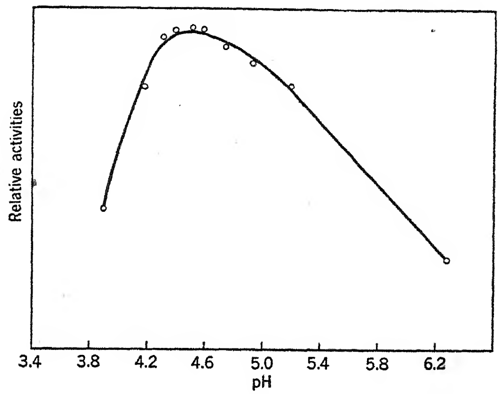

Fig. 4.—Influence of hydrogen-ion activity on the action of beta-amylase from malted barley. Purified amylase; 2 per cent soluble potato starch; 0.01 *M* acetate; 30 minutes at 40° C (Caldwell and Doebbeling, 1935).

Ascorbic acid has been reported to inhibit the action of the beta-amylase of barley and of malted barley (Purr, 1934; Hanes, 1935a; Weidenhagen, 1936; Janicki, 1939). Purr (1934) found that dehydroascorbic acid had no influence on beta-amylase. Weidenhagen (1936) found that the unfavorable influence of the ascorbic acid was diminished by the presence of glutathione. These relationships have not yet been satisfactorily explained.

The Dextrinogenic or Alpha-Amylase of Malted Barley

Action on Starches. In contrast to the beta-amylase, the dextrinogenic or alpha-amylase of malted barley causes marked decrease in the viscosities of starch pastes and rapid cleavage to products which give no color with iodine and which exhibit falling, or alpha-mutarotation (Kuhn, 1925; Ohlsson, 1930). While small concentrations of maltose have been reported (Freeman and Hopkins, 1936a; Samec, 1937), the reducing values of the hydrolysis mixtures of malt alpha-amylase, in the early stages of the reaction at least, are due largely to reducing substances of higher molecular weights than maltose. Different kinds of evidence support this statement.

The reducing values of the hydrolysis mixtures of malt alpha-amylase, when converted to maltose, differ when they are obtained by copper-reduction or by iodine-reduction methods (Blom *et al.*, 1936).

As already pointed out, such differences disclose the presence of reducing substances other than maltose.

The results of selective fermentation of the hydrolysis mixtures with yeasts led Hanes (1935b) to conclude that a considerable proportion of the reducing material formed from starch by malt alpha-amylase is neither maltose nor glucose.

Ohlsson (1930) used osmotic-pressure measurements to study the early products of the action of alpha-amylase from malted barley on soluble potato starch. In one case, the reaction was stopped when the reducing value was equivalent to 23 per cent of the theoretical yield of maltose and when the color with iodine was violet. After several days to allow for equilibrium to be attained, the pressure in the osmometer was still much higher for the reaction mixture (227 mm water) than for the unhydrolyzed substrate (48 mm water). The high value for the reaction mixture at equilibrium indicated that relatively large numbers of nondiffusible or slowly diffusible fragments of relatively high molecular weights had been formed. The nondiffusible fraction accounted for 80 per cent of the reducing value of the reaction mixture. The reducing values of the diffusible as well as those of the nondiffusible fractions indicated the presence of reducing substances of higher molecular weights than maltose.

Fractionation of the products formed in the early stages of the hydrolysis of starch by alpha-amylase from malted barley gave alcohol-insoluble fractions which accounted for 30 to 40 per cent of the reducing values of the reaction mixtures (Hanes, 1935b; Freeman and Hopkins, 1936a). The reducing values, optical rotation, and rates of fermentation with yeast, of the alcohol-soluble or sugar fractions, showed that they also contained significant concentrations of reducing substances of higher molecular weights than maltose (Freeman and Hopkins, 1936a).

A characteristic flocculation of the reaction mixtures occurs during the early stages of the hydrolysis of starches by malt alpha-amylase. Hanes (1935b) suggests that this flocculation may be due to the hydrolysis of a "supporting colloid." Recent work with amyloses and amylopectins suggests that the supporting colloid is the amylopectin and that the flocculation is due to the precipitation of the amylose as the amylopectin is hydrolyzed (Meyer, 1942; Wilson, Schoch, and Hudson, 1943).

When the achromic stage is reached in the hydrolysis of starches by malt alpha-amylase, the reducing action of the products is equivalent to approximately 30 per cent of the theoretical yield of maltose (van Klinkenberg, 1932b; Hanes, 1937; Samec and Blinc, 1939), and the reducing material appears to contain large proportions of dextrins made up of six or seven glucose units. This latter suggestion is based upon the work of a number of investigators. It is in accord with the average molecular weights

of the fractionated dextrins, with their reducing values and optical rotation (Brown and Morris, 1885; Ling and Baker, 1897; Brown and Millar, 1899; Prior and Wiegmann, 1900; Syniewski, 1902; Myrbäck, 1941a; Ohlsson, 1926; Örtenblad and Myrbäck, 1941a, 1943), with the glucose which the fractionated dextrins yield on hydrolysis (Hanes, 1935b), and with the evidence that chain fragments of more than six or seven glucose units are required to give a color with iodine (Waldschmidt-Leitz, Reichel, and Purr, 1932b; Haworth, Hirst, and Plant, 1935; Hanes, 1937; Freudenberg *et al.*, 1939, 1940a, b; Rundle and Baldwin, 1943; see also Meyer and Bernfeld, 1941a, and review articles by Samec and Blinc, 1939; Meyer, 1942;

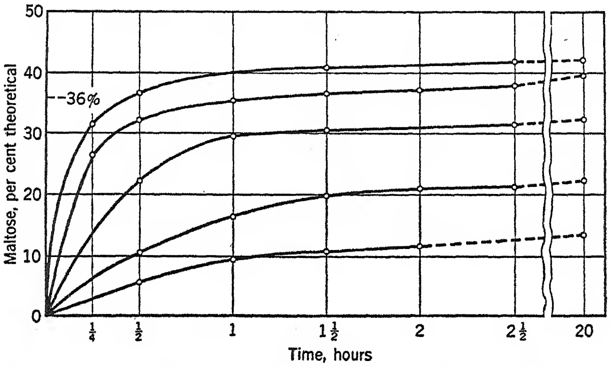

Fig. 5.—Influence of enzyme concentration on the action of alpha-amylase from malted barley. Soluble potato starch, 1 per cent; *p*H, 5.75; 0.025 *N* citrate; 40° C; purified alpha-amylase in relative concentrations of x, $2x$, $5x$, $12.5x$, and $20x$ (van Klinkenberg, 1932b).

Myrbäck, 1943). On the other hand, the evidence is not entirely conclusive and Hopkins, Dolby, and Stopher (1942) report that dextrins of six or seven glucose units do not predominate among the products obtained at the achromic stage in the hydrolysis of starch by malt alpha-amylase.

From a survey of the literature it is evident that further studies are needed of the products formed from starches and their components by amylases. The results obtained by the older criteria should be amplified and extended by the use of newer methods, such as end-group assay and centrifugal studies.

The data given in Figure 5 illustrate the action of malted barley alpha-amylase upon soluble potato starch (van Klinkenberg, 1932b). When sufficient amylase is used, the reducing action of the hydrolysis mixtures increases rapidly to a value which is equivalent to 30 to 40 per cent of the theoretical yield of maltose and then slowly to values which appear to depend upon the concentration of the amylase. The abrupt transition

from a rapid to a slow rate of reaction, and the variations in the extent of the hydrolysis with different concentrations of the enzyme, are typical of the results so far obtained with this amylase (Hanes, 1935b; Samec, 1937). By the use of relatively high concentrations of preparations of malt alpha-amylase and by prolonged periods of hydrolysis, much higher reducing values than those given in Figure 5 have been reported (Hopkins and Kulka, 1942; Kneen, 1943; see also summary by Samec and Blinc, 1939).

It seems significant that the achromic stage is reached with malt alpha-amylase at approximately the end of the rapid phase of the reaction (Hanes, 1937; Samec and Blinc, 1939). This correlation suggests that malt alpha-amylase hydrolyzes slowly dextrins of relatively low molecular weight. It is also quite possible that these dextrins are broken down, not by the alpha-amylase, but by traces of beta-amylase or of other enzymes present as impurities in the alpha-amylase preparations. The dependence of the limit of the hydrolysis upon the concentration of the alpha-amylase preparation (Fig. 5) would then be explained by the presence of traces of other enzymes which are also increased with increasing concentrations of the alpha-amylase preparation. Additional explanations present themselves. The slow phase of the reaction may be due to the partial inactivation of malt alpha-amylase (Hopkins and Kulka, 1942) or to the establishment of equilibria between the amylase or the substrate and the products of the hydrolysis. More evidence is needed to settle these questions.

Maltose and glucose have both been reported in significant amounts in the later stages of the action of malt alpha-amylase (Ohlsson, 1922; Hanes, 1935b; Samec, 1939; Myrbäck, 1941b; Örtenblad and Myrbäck, 1943; Hopkins, Dolby, and Stopher, 1942). Among other reducing sugars reported are isomaltose and tri- and tetrasaccharides made up of glucose units but containing an α-1,6-glucosidic linkage (Ling and Carter, 1938; Myrbäck and Ahlborg, 1940a; Myrbäck, 1943; Örtenblad and Myrbäck, 1943).

Mode of Action. The alpha-amylase of malted barley causes much more complete and rapid fragmentation of starches than the beta-amylase which hydrolyzes maltose from the ends of the glucosidic chains. While not entirely conclusive, several kinds of evidence indicate that dextrins made up of six or seven glucose residues predominate at the achromic stage of the hydrolysis. Hanes (1937) suggested that the formation of such dextrins might be explained if the glucose chains of starches were assumed to "exist in the form of close spirals, each coil of which would contain exactly six glucose units." He suggested that the "coils" would offer points of attack for the alpha-amylase throughout the molecule. Hanes' hypothesis appears plausible for the amylose components of starches. Several kinds

of evidence indicate that the glucosidic chains of the amyloses probably exist in the form of helices (Freudenberg, Schaaf, Dumpert, and Ploetz, 1939; Meyer, 1943; Rundle and Baldwin, 1943; Rundle and French, 1943). On the other hand, the amylopectin molecules with their many branches probably do not exist as helices. Therefore the "coils" suggested by Hanes do not give an entirely satisfactory explanation of the action of alpha-amylase on starches.

The amylopectin components of starches are rapidly hydrolyzed by alpha-amylase with no evidence of the formation of resistant dextrins of high molecular weights like those formed by beta-amylase. Alpha-amylase must therefore attack the α-1,4-glucosidic linkages between the branches of the amylopectin molecule as well as those of the outer branches (see Fig. 2). The possible presence in the later stages of the hydrolysis of di-, tri-, or tetrasaccharides, made up of glucose units but containing an α-1,6-glucosidic linkage, leaves doubt as to whether malt alpha-amylase hydrolyzes the α-1,6-glucosidic linkages of the amylopectin molecules (Myrbäck, 1938b, 1941a,b, 1943).

The liquefying action on starches of extracts of malted barley appears to be due largely to alpha-amylase activity. Kneen, Beckord, and Sandstedt (1941a) found excellent agreement between the alpha-amylase activity as determined by their dextrinogenic method for alpha-amylase (Sandstedt, Kneen, and Blish, 1939) and the capacity of extracts of malted barley to liquefy starch (Józsa and Johnston, 1935). They found that, with the ratios of malt extract to starch usually employed, the beta-amylase of the extract had no appreciable effect on the rate of the liquefaction of the starch. However, it must be remembered that other enzymes such as the amylophosphatase of Waldschmidt-Leitz and Mayer (1935) and glucosidases (Meyer, 1942; Meyer *et al.*, 1942a) may also be present in such extracts and may influence the liquefying as well as the other activities of the alpha-amylase.

Chemical Nature and Properties. Purified preparations of malt alpha-amylase have been obtained by a number of workers (van Klinkenberg, 1932a; Hanes, 1935b; Holmbergh, 1932, 1933a,b, 1935, 1938). Holmbergh (1933a) reported that starch could be used for the selective adsorption of malt alpha-amylase. The most highly active preparations of malt alpha-amylase so far reported are protein (Caldwell and Doebbeling, 1935, and subsequent work).

In crude aqueous extracts, malt alpha-amylase is usually much more thermostable than malt beta-amylase. Upon purification, however, this difference between the two amylases becomes less marked and dilute aqueous solutions of partially purified malt alpha-amylase can no longer be heated to remove traces of beta-amylase.

Malt alpha-amylase is readily inactivated when its solutions are adjusted to high hydrogen-ion activities. This property is marked even in crude aqueous extracts of malted barley and differentiates malt alpha-amylase from malt beta-amylase, which is much more stable under these conditions.

The evidence concerning the influence of cations and anions on the stability and activity of malt alpha-amylase is contradictory, probably because so many interrelated factors are involved. Some of these interrelationships have been studied in extracts of malted barley and of malted wheat by Kneen, Sandstedt, and Hollenbeck (1943b). Of special interest is

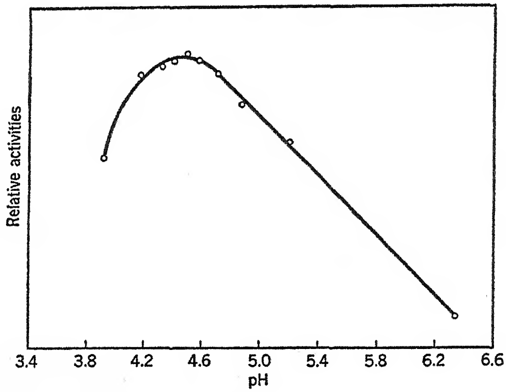

Fig. 6.—Influence of hydrogen-ion activity on the action of alpha-amylase from malted barley. Purified amylase; 2 per cent soluble potato starch; 0.01 *M* acetate; 30 minutes at 40° C Caldwell and Doebbeling, 1935).

their report that calcium ions exert a favorable influence upon the stability of malt alpha-amylase when its solutions are adjusted to high hydrogen-ion activities and also when they are held at high temperatures.

Ascorbic acid, dehydroascorbic acid, and glutathione have been reported to influence the stability and activity of malt alpha-amylase, but the evidence is not clear-cut (Pringsheim *et al.*, 1932; van Klinkenberg, 1932b; Purr, 1934; Weidenhagen, 1936; Janicki, 1939; Holmbergh, 1940).

The data given in Figure 6 illustrate the influence of the hydrogen-ion activity of its substrate upon the activity of purified malt alpha-amylase (Caldwell and Doebbeling, 1935). Somewhat different hydrogen-ion activities have been reported for the optimal action of malt alpha-amylase when it hydrolyzes starch under other conditions (Ohlsson, 1926, 1930; Waldschmidt-Leitz, Reichel, and Purr, 1932b; Holmbergh, 1932, 1933a,b; van Klinkenberg, 1932a, 1934; Hanes, 1935b).

Topics Relating to Both Alpha- and Beta-Amylases

Comparison of Stabilities of the Alpha- and Beta-Amylases of Malted Barley. In crude aqueous extracts, the beta-amylase of malted barley is much more thermolabile than the alpha-amylase. This was recognized by early investigators and was utilized by Ohlsson to remove beta-amylase from its mixtures with alpha-amylase. According to Ohlsson (1926, 1930), if crude aqueous extracts of malted barley, at their natural acidity, are held at 70° for 15 minutes, the beta-amylase will be completely inactivated while much of the alpha-amylase activity will remain. Many investigators have confirmed Ohlsson's findings; and this, or a similar procedure, is widely used to prepare alpha-amylase solutions from malted grains.

It must be emphasized, however, that the results obtained are not uniform. The concentrations of the amylases in the extracts, and the kinds and concentrations of substances which accompany them, influence the rate of inactivation of both amylases. Kneen, Sandstedt, and Hollenbeck (1943b), working with extracts of malted barley, found that calcium ions are of particular importance; they increase the thermolability of the beta-amylase and decrease that of the alpha-amylase. Upon purification, both amylases become much more thermolabile; not only highly purified malt beta-amylase but also highly purified malt alpha-amylase is completely inactivated when held in dilute aqueous solution for one minute at 50° (Caldwell and Doebbeling, 1935).

The alpha-amylase in extracts of malted barley is more sensitive to high hydrogen-ion activities than the beta-amylase. Ohlsson (1930) utilized this property to remove alpha-amylase from its mixtures with beta-amylase. If an extract of malted barley is acidified to *p*H 3.3, held at 0° for 15 minutes, and then neutralized, it will exert beta-amylase, but little if any alpha-amylase, activity. These results also depend upon many interrelated factors, and the complete inactivation of the alpha-amylase cannot be assumed simply because the extract has received the prescribed treatment. Kneen *et al.* (1943b) found that, here again, calcium ions are important; they decrease the loss of alpha-amylase activity and increase the loss of beta-amylase activity in the presence of unfavorable hydrogen-ion activities. Upon purification, the beta-amylase also becomes increasingly sensitive to high hydrogen-ion activities and can no longer be separated from the alpha-amylase by the above procedure (Caldwell, unpublished).

Joint Action of the Alpha- and Beta-Amylases of Malted Barley. The joint action of the alpha- and beta-amylases of malted barley causes more rapid and extensive hydrolysis of starch than equivalent concentrations of either amylase alone. This is readily explained by the findings

that the products formed by each of these amylases are subject to further hydrolysis by the other. Meyer (1942) reports that the residual dextrin formed from starch by beta-amylase is attacked by alpha-amylase, by alpha-glucosidase, and by superheated water, and that the resulting products are further broken down by beta-amylase (see also Hanes, 1937; Meyer and Bernfeld, 1942a). Myrbäck (1941a) reports that beta-amylase rapidly hydrolyzes to maltose the dextrins with molecular weights of approximately 1000 produced from starch by malt alpha-amylase. If, then, a beta-amylase preparation contains sufficient alpha-amylase or other carbohydrase to keep hydrolyzing α-1,4-glucosidic linkages between branches of the amylopectin molecules, new free α-1,4-glucosidic chains are made available for the further action of beta-amylase and a high degree of hydrolysis is attained.

The main product of the hydrolysis of starches by mixtures of the alpha- and beta-amylases of malted barley is maltose. Some glucose is also formed (Hanes, 1937; Samec and Blinc, 1939). In addition, isomaltose, and tri- and tetrasaccharides with α-1,6-glucosidic as well as α-1,4-glucosidic linkages, have been reported (Ling, Carter, and Potter, 1938; Ling and Carter, 1938; Myrbäck *et al.*, 1940a,c; Myrbäck, 1941b, 1943).

Differentiation. It is relatively easy to prove the presence or absence of a dextrinogenic component in an amylase preparation. The dextrinogenic amylases are differentiated from the saccharogenic amylases by the characteristic changes in the color given with iodine by their reaction mixtures, by the rapid disappearance of products which give color with iodine, and by the rapid decrease in the viscosities of their reaction mixtures. If these characteristic changes all occur, a dextrinogenic component is present. If they do not take place even when widely different concentrations of the amylase preparation are used, a dextrinogenic component can be judged to be absent or negligible.

The hydrolysis of starch by alpha-amylase is influenced by the presence of beta-amylase. Although beta-amylase alone does not hydrolyze starch to products which give no color with iodine, the achromic stage is reached more rapidly in the presence of both alpha- and beta-amylases than in the presence of alpha-amylase alone (Ohlsson, 1926, 1930; Holmbergh, 1933b; Blom, Bak, and Braae, 1937; Hanes and Cattle, 1938). Sandstedt, Kneen, and Blish (1939) have modified the Wohlgemuth (1908) method to give a quantitative determination of alpha-amylase in the presence of excess beta-amylase under conditions which make alpha-amylase the limiting factor.

The method proposed by Wijsman (1889, 1890) forms the basis for procedures which are being widely used in attempts to prove that amylase preparations contain both alpha- and beta-amylases or either one alone. These methods depend upon the difference in the rates of diffusion of the

two amylases in stiff gelatin jellies which contain small concentrations of soluble starch. The diffusion of the amylases may be detected after a period of time by noting the colors formed when the gelatin–starch–amylase jelly is treated with dilute iodine.

A colorless central zone on such a diffusion plate is evidence of the presence of an alpha-amylase because beta-amylase alone does not hydrolyze starch to the achromic stage. On the other hand, a blue-violet outer zone surrounded by the deep blue of the unchanged starch is supposed to indicate the presence of beta-amylase.

Because alpha-amylase hydrolyzes starch to products which show a gradation of colors with iodine, and because the blue-violet shades obtained cannot be distinguished from those given by the products of beta-amylase action, a transplanting technique was included by Wijsman to confirm the presence of beta-amylase. Duplicate plates of gelatin–starch jelly are inoculated with the amylase. After the reaction period, one plate is treated with iodine and the colors noted. The position on the duplicate plate which corresponds to that which gave a blue-violet color with iodine on the first plate is noted. A block of starch–gelatin–amylase jelly is then taken from this position in the duplicate plate and used to inoculate a third gelatin–starch plate with amylase. After sufficient time has been allowed for the amylase to diffuse into the starch–gelatin jelly, the third plate is treated with iodine; and, if another blue-violet ring develops with no colorless central zone, the presence of beta-amylase free from alpha-amylase in the outer zone of the first plate is assumed to have been demonstrated.

If both amylases are present in relatively large concentrations, this procedure is qualitatively satisfactory. It was originally proposed by Wijsman to show that there are two amylases in extracts of malted barley. The procedure may show that beta-amylase preparations are free from alpha-amylase provided a wide range in the concentration of the amylase is employed and no colorless zone is obtained. The procedure cannot be used to show that alpha-amylase preparations are free from traces of beta-amylase because the appearance of blue-violet zones may indicate merely that the transplanting technique gives insufficient alpha-amylase to hydrolyze all of the starch at any given point to the achromic stage.

Hanes and Cattle (1938) use a spectrophotometric procedure for the quantitative study of the alterations in color given with iodine by the products of the hydrolysis of starches by amylases. The changes in the absorption characteristics of the iodine complexes are quite different for the products formed from starch by dextrinogenic and by saccharogenic amylases and make it possible to distinguish between preparations in which one or the other of these amylases predominates.

Starch liquefaction or the rapid decrease in the viscosities of starch pastes which is characteristic of the action of alpha-amylase has been proposed as the basis of methods for the quantitative determination of alpha-amylase activity (Józsa and Johnston, 1935; Blom, Bak, and Braae, 1937). Kneen, Beckord, and Sandstedt (1941a) and Hollenbeck and Blish (1941) found that the liquefying action (Józsa and Johnston, 1935) of malt extracts closely parallels the alpha-amylase activity obtained by the alpha-amylase dextrinization (iodine color) method of Sandstedt, Kneen, and Blish (1939). The results showed that, under the conditions usually employed, the beta-amylase in extracts of malted barley does not appreciably influence the viscosity measurements obtained.

Alpha-mutarotation of hydrolysis mixtures indicates the presence of an alpha-amylase but does not prove the absence of beta-amylase, while beta-mutarotation of hydrolysis mixtures proves the presence of a beta-amylase but not the absence of an alpha-amylase. Kuhn (1925) and Ohlsson (1930) reported beta-mutarotation in the hydrolyzates of extracts of malted barley which contain significant concentrations of alpha-amylase.

Increases in the reducing values of reaction mixtures are obtained in the hydrolysis of starches by both dextrinogenic and saccharogenic amylases. Therefore, it is not possible to use the determination of saccharogenic activities alone to distinguish between these two kinds of amylases.

Kneen and Sandstedt (1941b) report that the saccharogenic activities of the alpha- and beta-amylases of malted barley and of malted wheat are additive within certain rather wide limits (ratios of beta-amylase to alpha-amylase of 50:1 to 0.05:1) and have developed a method for the quantitative determination of beta-amylase in the presence of alpha-amylase. The alpha-amylase is determined by the modified Wohlgemuth method given above (Sandstedt, Kneen, and Blish, 1939). This value is then converted to its equivalent in saccharogenic activity by reference to the relationship of alpha-saccharogenic to alpha-dextrinogenic activities as established with a preparation of alpha-amylase. The saccharogenic activity of beta-amylase is then calculated by subtracting the saccharogenic value due to the alpha-amylase from the total saccharogenic value.

The marked changes in the ratios of the saccharogenic to the dextrinogenic activities found when extracts of malted barley are heated or acidified led to the recognition of two amylases in these extracts. Conversely, relatively small changes in these ratios are often taken as proof that only one amylase is present. However, measurements of amylase activities are not yet sufficiently precise to eliminate the possibility of the presence of traces of a second amylase.

The limitation of the hydrolysis of soluble potato starch at approxi-

mately 64 per cent of the theoretical maltose and the persistence of a blue-violet or violet color with iodine after the prolonged action of the amylase, together, give convincing evidence that a beta-amylase preparation is free from traces of alpha-amylase.

No methods are yet available to give convincing evidence for the absence of beta-amylase activity in an alpha-amylase preparation. It is important to remember that the action attributed to alpha-amylase preparations from malted grains may often be influenced by traces of beta-amylase or of other enzymes (Ohlsson, 1926, 1930; Holmbergh, 1933b; Blom, Bak, and Braae, 1937; Hanes and Cattle, 1938; Meyer and Bernfeld, 1942a,b).

Occurrence. Beta-amylase is the predominant amylase of sound ungerminated barley. Alpha-amylase does not appear to be present in active form in appreciable amounts in such grains (Nordh and Ohlsson, 1932; Lüers and Rümmler, 1933, 1935). However, extracts of barley intended for the study of beta-amylase activity alone should be examined for alpha-amylase activity (Hills and Bailey, 1938b; Kneen, Sandstedt, and Hollenbeck, 1943b; Kneen, 1944). Upon the germination and malting of barley, the available or measurable beta-amylase is markedly increased and alpha-amylase is formed or set free. Numerous investigators have reported that extracts of germinated or malted barley exhibit both alpha- and beta-amylase activities.

It is probable that proteases play an important part in the changes which take place in the amylase activities during the germination and malting of barley. This influence is indicated by the marked increases in amylase activities obtained in extracts of barley, germinated barley, or malted barley, by the aid of proteases like papain (Ford and Guthrie, 1908a,b; Baker and Hulton, 1922; Myrbäck and Myrbäck, 1933, 1936; Lüers and Lechner, 1933; Chrzaszcz and Janicki, 1933; Myrbäck and Örtenblad, 1937–38; Hills and Bailey, 1938a,b; Dull and Swanson, 1941; Kneen, Beckord, and Sandstedt, 1941a). The proteases may free the active amylases from some inactive complex or may bring about changes from an inactive to an active protein analogous to those which transform inactive pepsinogen, trypsinogen, or chymotrypsinogen, into the corresponding active enzymes, pepsin, trypsin, or chymotrypsin (Northrop, 1937). The increased amylase activities of germinated grains and those obtained by the use of proteases have also been explained by the postulated liberation of activators or suppression of inhibitors (Waldschmidt-Leitz and Purr, 1931; Lüers and Lechner, 1933; Lüers, 1935, 1936; Chrzaszcz and Janicki, 1933, 1936; Chrzaszcz and Swiatkowska, 1937; Snider, 1940).

Proteases like papain are extensively used in industry to increase the amylase activity of extracts of barley and of malted barley. The "total"

amylase activity thus obtained is usually measured saccharogenically. By the use of their methods for the quantitative determination of alpha-amylase or beta-amylase in mixtures of the two (Sandstedt, Kneen, and Blish, 1939; Kneen and Sandstedt, 1941b), Kneen, Beckord, and Sandstedt (1941a) have shown that the increase in saccharogenic activity which results from protease treatment is due to increases in the activities of both the alpha- and the beta-amylases. These authors also find that the relative increases in alpha- and beta-amylase activities after the use of a protease like papain depend upon the particular barley used.

Many factors influence the amylase content of barley and of malted barley. Important among these are the variety of the seed and the environment of its growth. Studies of these factors have disclosed correlations and complex interrelationships between the active amylases and other properties of the seed. It is impossible to summarize here the findings of great practical importance and theoretical significance which have been reported. Summaries of their observations are given by a number of workers (Dickson, Dickson, Shands, and Burkhart, 1938; Hills and Bailey, 1938b; Anderson, Sallans, and Meredith, 1941; Dickson and Burkhart, 1942; Kneen, 1943).

Another phase of the general subject of the occurrence of alpha- and beta-amylases of theoretical interest and promise deals with the study of the individual grains to determine the kind of changes and the seat of the changes which accompany the formation and liberation of the active amylases (Linderstrøm-Lang and Engel, 1937; Ohlsson and Thörn, 1938; Dickson and Burkhart, 1942).

Purification and Separation of Alpha-Amylase and Beta-Amylase from Malted Barley. The amylases of malted barley have been purified by many procedures. These include alcohol precipitation (Lintner, 1886, 1887; Osborne, 1895; Osborne and Campbell, 1896; Sherman and Schlesinger, 1915a; Ling and Nanji, 1923, 1925; van Klinkenberg, 1932a; Hopkins, Cope, and Green, 1933; Hanes, 1935b), precipitation by ammonium sulfate (Osborne, 1895; Sherman, Caldwell, and Doebbeling, 1934; Caldwell and Doebbeling, 1935; Kneen, Sandstedt, and Hollenbeck, 1943b), precipitation by barium hydroxide (Holmbergh, 1938), precipitation by tannin (Weidenhagen, 1933), and many kinds of adsorption techniques (Lüers and Sellner, 1925; Samec and Waldschmidt-Leitz, 1931; Waldschmidt-Leitz, Reichel, and Purr, 1932b; Holmbergh, 1932, 1933a,b, 1935, 1938).

Holmbergh (1932, 1933a,b) reports the use of starches for the differential adsorption of malt alpha-amylase. He also reports (1938) the preparation of what appears to be exceedingly active beta-amylase. He used fractional precipitation with barium hydroxide, hydrogen sulfide, and ethanol. The products exerted high saccharogenic activities. The report

available to the reviewers does not state whether the final products were examined for or showed alpha-amylase activity.

Preparations of beta-amylase of exceedingly high activities have been obtained from aqueous extracts of malted barley by repeated fractionation with ammonium sulfate, dialyses, and fractionation with ethanol (Sherman, Caldwell, and Doebbeling, 1934; Caldwell and Doebbeling, 1935, and subsequent work). The repeated fractionation with ammonium sulfate, with intervening dialyses of the aqueous solutions of the precipitates, yields preparations of beta-amylase with negligible dextrinogenic activity* and with a saccharogenic activity† of approximately 5000 in contrast to 100 in the crude extract. Further fractionation of this highly active material with ethanol and ether results in preparations with saccharogenic activities of approximately 13,000 which give no evidence of alpha-amylase activity as measured by the modified Wohlgemuth method used on a micro scale (Sherman and Thomas, 1915b). The hydrolysis mixtures obtained with these preparations continue indefinitely to give a blue-violet color with iodine; the limit in the hydrolysis of soluble potato starch is approximately 64 per cent of the theoretical yield of maltose.

Preparations of alpha-amylase are also obtained from extracts of malted barley by repeated fractionation with ammonium sulfate with intervening dialyses. The alpha-amylase tends to be separated in the earlier stages of the fractionation and by lower concentrations of the salt than are required for the precipitation of the beta-amylase. Typical preparations of alpha-amylase have a saccharogenic activity of approximately 2500 and a dextrinogenic activity of approximately 10,000. The ratio of saccharogenic to dextrinogenic activities of 0.25 is similar to that given by alpha-amylase preparations obtained by alcohol fractionation of extracts of malted barley which have been heated to remove beta-amylase (Caldwell, unpublished). The purified preparations of both the alpha- and the beta-amylases are protein.

AMYLASES OF OTHER HIGHER PLANTS

Other cereal grains contain amylases which appear to be similar to, if not identical with, the amylases of barley. Beta-amylase has been reported in dormant, germinated, and malted grains which include oats, rice, rye, and wheat (Bailey, 1925; van Klinkenberg, 1932a; Ohlsson and Ed-

* The dextrinogenic activity represents the weight of starch hydrolyzed per unit weight of amylase preparation, under certain closely specified conditions, to products which give a clear red color with iodine.

† The saccharogenic activity represents the weight of "maltose" formed from starch per unit weight of amylase preparation under certain closely specified conditions.

feldt, 1933; Ohlsson and Uddenberg, 1933; Tychowski, 1937; Venkata-Giri and Sreenivasan, 1937; Stamberg and Bailey, 1938; Kneen, 1944); alpha-amylase has been reported after germination or malting in maize, millet, oats, rye, sorghum, and wheat (Baker and Hulton, 1921, 1929; Bailey, 1925; Naylor, Spencer, and House, 1925; Pronin, 1931; Chrzaszcz and Janicki, 1933; Ohlsson and Edfeldt, 1933; Ohlsson and Uddenberg, 1933; Andrews and Bailey, 1934; Chrzaszcz and Janicki, 1936; Venkata-Giri and Sreenivasan, 1937; Yamagishi, 1937; Stamberg and Bailey, 1938; Bernstein, 1943a,b; Kneen, 1944).

The detailed studies of the amylases of wheat are of special interest. With wheat as with barley, beta-amylase is the predominant amylase of the ungerminated grain while both alpha- and beta-amylases are found after germination and malting (Blish, Sandstedt, and Kneen, 1938; Kneen and Sandstedt, 1941b; Kneen, Miller, and Sandstedt, 1942; Kneen, 1944).

A number of investigators have studied the purification and properties of the amylases of wheat and of malted wheat (Lintner, 1887). Weidenhagen (1933) used precipitation with tannin, and van Klinkenberg (1932a) fractional precipitation with ethanol for the purification of beta-amylase from ungerminated wheat. Creighton and Naylor (1933) obtained preparations of purified beta-amylase by dialysis and fractional precipitation with ethanol of extracts of germinated wheat which had been acidified to inactivate the alpha-amylase (Ohlsson, 1930). Similarly, they obtained preparations of purified alpha-amylase by dialysis and fractionation with ethanol of extracts of germinated wheat after treatment to inactivate the beta-amylase (Ohlsson, 1930). Blish, Sandstedt, and Kneen (1938) used ammonium sulfate precipitation for the purification of wheat beta-amylase from extracts of soft wheat flour which had been acidified to inactivate the alpha-amylase (Ohlsson, 1930).

The alpha- and beta-amylases of wheat appear to be similar to the alpha- and beta-amylases of barley. Kneen and Sandstedt (1941b) and Kneen (1944) report that the saccharogenic potentials of the alpha-amylases from malted wheat and from malted barley are the same and that the saccharogenic activities of the alpha- and beta-amylases are additive within wide limits in both cases. Blish, Sandstedt, and Kneen (1938) found that wheat beta-amylase, like barley beta-amylase, reaches a limit in the hydrolysis of soluble starch at approximately 64 per cent of the theoretical maltose. Kneen, Sandstedt, and Hollenbeck (1943b) report that calcium ions increase the stability of wheat alpha-amylase as well as that of barley alpha-amylase in aqueous solutions held at unfavorable hydrogen-ion activities. These authors also found that calcium ions increase the thermolability of wheat beta-amylase as well as that of barley beta-amylase.

Certain differences in the stabilities of wheat and barley alpha-amylases and of wheat and barley beta-amylases observed in extracts of malted wheat and of malted barley may represent true differences in the properties of the alpha- or of the beta-amylases from these two sources, or may be due to the influence of extraneous substances in the extracts studied.

Creighton and Naylor (1933) report that wheat beta-amylase exerts its optimal activity at *p*H 4.9 to 5.3 when it acts on 2 per cent soluble potato starch (0.02 to 0.06 *M* phosphate) for 30 minutes at 40°C; under similar conditions, but with one per cent starch, wheat alpha-amylase exerts its optimal dextrinogenic activity at *p*H 4.6 to 6.3.

Ballou and Luck (1941) studied the influence on wheat beta-amylase of ten buffers in systems of a constant ionic strength of 0.05 with 1 per cent starch adjusted to hydrogen-ion activities between *p*H 3.8 and *p*H 6.2. The measurements were made at 30°C. The buffers were formate, acetate, propionate, butyrate, valerate, phenyl acetate, phthalate, succinate, phosphate, and citrate. The variations in the buffer anions were without significant influence on the relative activities of the beta-amylase at the optimal hydrogen-ion activities, except for a slight inhibitory effect of phenyl acetate and phthalate. The enzyme activity–*p*H curves coincided on the alkaline side of the optima; on the acid side, a marked specific buffer influence was manifested.

Kneen (1944) has made a comparative study of the amylase activities of a number of germinated and ungerminated cereal grains. His survey of the literature and his own observations indicate that all germinated cereal grains are not good sources of both alpha- and beta-amylases but that in some cases the saccharogenic activity observed is due largely to alpha-amylase. He used the modified Wohlgemuth method of Sandstedt, Kneen, and Blish (1939) to measure the alpha-amylase activities and that of Kneen and Sandstedt (1941b) to distinguish between the total saccharogenic activities of the extracts and their beta-amylase activities. He has developed and applied a micro method for the measurement of beta-amylase activity. The calculations of the beta-amylase activities as distinguished from the total saccharogenic activities are based upon the assumption that the saccharifying potentials of the alpha-amylases of all the cereal grains studied are similar to that established for wheat and for barley (Kneen and Sandstedt, 1941b). This has been verified for sorghum (Kneen, 1944).

Kneen (1944) reports that beta-amylase is relatively high in ungerminated barley, wheat, and rye; much lower in ungerminated oats; not detectable by the method used in ungerminated maize, sorghum, or rice. All of the ungerminated grains studied gave evidence of low but measurable alpha-amylase activity when examined by the micro method (modified Wohlgemuth) of Kneen, Sandstedt, and Hollenbeck (1943b).

A study of the activities of the "free" amylase in calcium acetate extracts and of the "total" amylase in papain-treated extracts of the germinated grains showed that in all cases germination was coincident with increased alpha-amylase activity. The beta-amylase of barley, wheat, and rye became more readily extractable upon the germination of the grain. Beta-amylase activity was not detectable, by customary methods, in germinated oats, maize, or sorghum and was very low in germinated rice.

By taking into consideration the loss of dry weight which occurs during the germination of the grain, Kneen, Miller, and Sandstedt (1942) found that there is an apparent increase in the total (papain treatment) beta-amylase activity during the initial stages of the germination of wheat, followed by a pronounced and progressive decrease. Similarly, Kneen (1944) reports marked losses in the "absolute" values for the beta-amylase activities during the germination of wheat, barley, and rye, with simultaneous and progressive increases in the total alpha-amylase activities. Such results lead to such questions as: Is the beta-amylase necessary to the germination process? Does the loss or inhibition of the beta-amylase indicate that alpha-amylase is formed from beta-amylase? At present these questions remain unanswered.

While the concentration of amylase in cereal grains depends upon the variety and the environment of growth, among other factors, Kneen (1943) concludes from his comparative study that, on the average, barley grain tends to have more amylase than wheat but that wheat has a greater capacity for producing alpha-amylase during germination.

A great variety of other higher plants in addition to the cereal grains have been examined for amylase action. Among the studies which have differentiated between alpha- and beta-amylase activities is that of Venkata-Giri (1934) who found beta-amylase but no alpha-amylase in sweet potato tuber, *Ipomea batatas.* Borchardt and Pringsheim (1931) report that both alpha- and beta-amylases are present in the tuber of ordinary potatoes, *Solanum tuberosum.*

Beta-amylase appears to be the chief and perhaps the sole amylase of soybeans. Teller (1936) reported alpha-amylase in soybeans, but Newton and Naylor (1939) found only a possible trace of alpha-amylase activity (Wohlgemuth) either before or after germination. However, they report that, although their purified beta-amylase preparations exhibited "approximately no alpha-amylase activity" as determined by the Wohlgemuth method, they did exhibit marked ability to reduce the viscosity of starch pastes. The authors attribute the liquefying ability to the beta-amylase. The possibility of a trace of another enzyme such as amylophosphatase (Waldschmidt-Leitz and Mayer, 1935) does not appear to have been excluded. Orestano (1933) also concludes that beta-amylase is the sole

amylase of soybeans. In a later paper, Newton, Hixon, and Naylor (1943) describe the further purification of soybean beta-amylase. The highly active preparations obtained produced approximately 4600 times their weight of maltose in 30 minutes at 40°C from 2 per cent starch at *p*H 5.0.

The authors report a series of observations which indicate that soybean beta-amylase is much less thermolabile than the beta-amylase from barley or from wheat. The amylase was not entirely inactivated when dry ground soybeans were held at 100° for ten days. Even after purification, the amylase is remarkably stable in aqueous solution. Samples of six purified preparations of soybean beta-amylase were dissolved in distilled water, held at 25 to 30°C, and tested for amylase activity. The solutions of the three most active preparations retained 79, 76, and 62 per cent of their respective activities after 34 days. The three less purified preparations lost their activities much more rapidly and were inactive after 30 days. These results indicate that the loss of activity was influenced by some extraneous agent. In another series of experiments, soybean beta-amylase was allowed to react with cornstarch for 30 minutes at 76 to 78°C. The reducing value obtained in the reaction mixture corresponded to more than 70 per cent of that found when the amylase reacted with the starch at 40°C. On the other hand, soybean beta-amylase is not entirely thermostable. A dry purified preparation was completely inactivated when held at 200°C for 30 minutes and soybeans roasted commercially for sale as a confection contained no traces of active amylase.

PANCREATIC AMYLASE

Action on Substrates. Pancreatic amylase is a dextrinogenic alpha-amylase. It causes rapid decrease in the viscosity of starch pastes and rapid hydrolysis of starches to products which give no color with iodine and which exhibit alpha-mutarotation (Kuhn, 1924; Freeman and Hopkins, 1936c).

Although small concentrations of maltose (Freeman and Hopkins, 1936a) and of glucose (Sherman and Punnett, 1916a) have been reported in the early stages of the hydrolysis of starch by pancreatic amylase, dextrins and sugars of higher molecular weights than maltose appear to account for most of the reducing action of the products formed, at least until the achromic stage of the hydrolysis is reached. This conclusion is based on the reducing values, optical rotation, and rates of fermentation of the alcohol-soluble and alcohol-insoluble fractions separated from the hydrolysis mixtures (Freeman and Hopkins, 1936a,b,c).

When pancreatic amylase reacts with soluble potato starch, the

reducing values of the reaction mixtures rise rapidly and are equivalent to 50 to 55 per cent of the theoretical maltose at the achromic stage (Hanes, 1937; Hanes and Cattle, 1938; Freeman and Hopkins, 1936a). This relationship is in marked contrast to that observed for the action of malted barley alpha-amylase, which brings its reaction mixtures to the achromic stage at approximately 30 per cent of the theoretical maltose. This dissimilarity indicates that the hydrolysis of starch is brought about in different ways by these two alpha-amylases. However, sufficient evidence is not yet available concerning the early products formed by either amylase to warrant definite conclusions about the difference.

Glock (1936) and Myrbäck (1943) report that pancreatic amylase, like malt alpha-amylase, causes the rapid formation of reducing dextrins in which complexes composed of six glucose units predominate. These findings are supported by the work of Waldschmidt-Leitz and Reichel (1934), who obtained a crystalline dextrin made up of six glucose residues from among the products of the hydrolysis when pancreatic amylase reacted with an erythrodextrin which gave a red color with iodine.

The extent of the hydrolysis of starches by pancreatic amylase, and the products formed in the later stages of its action also, are not yet fully established. Willstätter *et al.* (1923), using soluble potato starch as substrate, found that the reducing values reach a limit at approximately 75 per cent of the theoretical maltose. This value has been confirmed by other workers (Blom, Bak, and Braae, 1937). Somewhat lower and higher values have been reported (Pringsheim and Leibowitz, 1926; Vonk and Braak, 1934; Pringsheim and Ginsburg, 1935; Freeman and Hopkins, 1936c). Myrbäck, Örtenblad, and Ahlborg (1940b,c) report that, in the later stages of the hydrolysis of starch by pancreatin, tri- and tetrasaccharides are present in considerable amounts. These sugars give evidence of α-1,6- as well as of α-1,4-glucosidic linkages.

Barbour (1929) reports that isomaltose is one of the products of the hydrolysis of glycogen by pancreatic amylase. This finding is of particular interest in connection with the very highly branched structure of the glycogen molecule.

Conditions which Favor the Stability and Activity. An outstanding characteristic of pancreatic amylase is its extreme sensitivity to its chemical environment (Sherman, Kendall, and Clark, 1910; Willstätter, Waldschmidt-Leitz, and Hesse, 1923). When the amylase and its substrate are both highly purified, pancreatic amylase exerts negligible activity until a salt such as sodium chloride is added (Kendall and Sherman, 1910). The kind and concentration of salt and the hydrogen-ion activities of the reaction mixtures are closely interrelated in their influence upon the action of pancreatic amylase.

The data given in Tables I to IV and in Figure 7 summarize the results of an extensive investigation of this interrelationship (Sherman, Caldwell, and Adams, 1928a,b,c). The reaction mixtures were maintained at the desired hydrogen-ion activities by the presence of 0.01 *M* phosphate, which had been found to have no influence on the action of pancreatic amylase in measurements of 30 minutes at 40°C with 1 or 2 per cent soluble potato starch (Sherman, Caldwell, and Dale, 1927b).

In order to establish the conditions which would permit the amylase to exert the highest activity of which it is capable in 30 minutes at 40°C in the presence of a given salt, each salt was studied at a number of different concentrations and each concentration over a wide range of hydrogen-ion activities.

The data summarized in Table I show that the optimal hydrogen-ion activities may differ widely in the presence of different concentrations of the same salt.

TABLE I

DATA SHOWING MOST FAVORABLE HYDROGEN-ION ACTIVITY (*p*H) FOR PANCREATIC AMYLASE IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF VARIOUS SALTS (From Sherman, Caldwell, and Adams, 1928a)

Data for 2 per cent soluble potato starch, 0.01 *M* phosphate, 30 minutes at 40°C

Concentration of salt, *M*	Sodium chloride	Potassium chloride	Sodium bromide	Sodium nitrate	Sodium chlorate	Sodium thiocyanate	Sodium fluoride
0.0005	6.5	—	—	—	—	—	—
0.001	6.7	—	—	—	—	—	—
0.0025	6.9	—	—	—	—	—	—
0.005	7.0	7.0–7.1	—	6.6–6.8	6.5	—	—
0.01	7.1	7.1–7.2	7.1	6.9–7.1	—	—	—
0.02	7.1	—	—	—	—	—	—
0.03	7.1	7.1–7.2	—	—	—	—	—
0.05	7.1	7.1–7.2	7.1	7.0–7.2	6.9–7.1	6.5	—
0.10	7.1	—	7.1	—	—	6.7–6.8	6.3–6.7
0.15	—	—	—	—	—	6.7–6.8	—
0.20	—	—	7.1	7.1–7.2	6.9–7.1	6.7–6.8	6.6–6.8
0.30	—	—	—	—	—	—	6.6–6.8

Comparative measurements of amylase activity in the presence of different concentrations of a given salt, with the reaction mixture adjusted in each case to the most favorable hydrogen-ion activity, disclosed the most favorable concentration of the salt under consideration. The results of such studies are summarized in Table II.

For each of the salts studied, the hydrogen-ion activity for the optimal amylase action decreases as the concentration of the salt increases until sufficient of the salt is present to permit maximum amylase activity.

TABLE II

THE OPTIMAL CONCENTRATIONS OF CERTAIN SALTS WITH THE CORRESPONDING OPTIMAL HYDROGEN-ION ACTIVITIES FOR THE ACTIVITY OF PANCREATIC AMYLASE
(From Sherman, Caldwell, and Adams, 1928b)

Data for 2 per cent soluble potato starch, 0.01 *M* phosphate, and 30 minutes at 40°C

Salt	Optimal concentration, *M*	Optimal *p*H
Sodium chloride	0.02–0.05	7.1–7.2
Potassium chloride	0.03–0.05	7.1–7.2
Sodium bromide	0.03–0.20	7.1
Sodium nitrate	0.10–0.20	7.1
Sodium chlorate	0.10–0.20	6.9–7.1
Sodium thiocyanate	0.15–0.20	6.7–6.8
Sodium fluoride	0.20–0.30	6.7–6.8

After the amylase has been fully activated by a given salt, the addition of a considerable excess of that salt causes no further change either in the activity of the amylase (Fig. 7) or in the optimal hydrogen-ion activity (Table I).

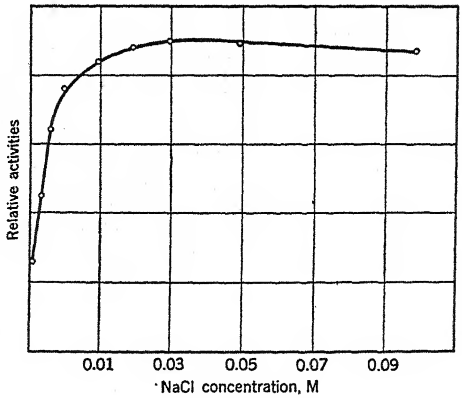

Fig. 7.—Influence of concentration of sodium chloride on the activity of pancreatic amylase. Purified amylase; 30 minutes at 40° C; 2 per cent soluble potato starch; 0.01 *M* phosphate; optimal *p*H in each case, see Table I (Sherman, Caldwell, and Adams, 1928b).

Comparisons of the activity of pancreatic amylase in the presence of the different salts, each at the most favorable concentration and hydrogen-ion activity, are summarized in Table III. The data in this table show that, even when comparisons are made under the best conditions for the enzyme in the presence of each salt, marked differences in the activity of the amylase are observed; the data show clearly that anions exert a specific influence on the activity of pancreatic amylase and that the chloride ion is the most effective activator.

TABLE III

COMPARISON OF THE ACTIVITY OF PANCREATIC AMYLASE IN THE PRESENCE OF VARIOUS SALTS, EACH AT THE OPTIMAL HYDROGEN-ION ACTIVITY AND CONCENTRATION (From Sherman, Caldwell, and Adams, 1928c)

Data for 2 per cent soluble potato starch, 0.01 *M* phosphate, and 30 minutes at 40 °C

Salt	Concentration of salt, *M*	Reaction mixture, *p*H	Pancreatin	Purified amylase
Sodium chloride	0.03 (standard)	7.1	100	100
Potassium chloride	0.03	7.1	100	100
Lithium chloride	0.02–0.05 (no phosphate)	7.1	80–90	80–90
Sodium bromide	0.03	7.1	77	77
Sodium nitrate	0.10	7.1	41	40
Sodium chlorate	0.10	6.9	29	27
Sodium thiocyanate	0.15	6.7	29	28
Sodium fluoride	0.20	6.7	24	21
Sodium sulfate	0.01–0.10	5.7–7.7	0	0
Sodium phosphate	0.01	5.7–7.7	0	0

If, in addition to the chloride ion, another anion such as the nitrate ion is also present, the activity of the amylase is reduced to an extent which depends upon the relative concentrations of the two (Table IV).

TABLE IV

INFLUENCE OF THE CONCENTRATION OF SODIUM NITRATE ON THE ACTIVITY OF PANCREATIC AMYLASE IN THE PRESENCE OF SODIUM CHLORIDE (From Sherman, Caldwell, and Adams, 1928c)

Data for 2 per cent soluble potato starch, 0.01 *M* phosphate, and 30 minutes at 40 °C

Sodium nitrate concentration, *M*	Sodium chloride concentration, *M*	Reaction mixture, *p*H	Pancreatin	Purified amylase
0	0.02	7.1	100	100
0.01	0.02	7.1	92	91
0.10	0.02	7.1	68	66
0.20	0.02	7.1	54	55
0.10	0	7.1	41	40
0	0	7.1	—	Negligible

As is shown in Table III, no measurable amylase activity was obtained in the presence of sulfate or of phosphate for the concentrations of pancreatic amylase used in these investigations. When the concentration of the enzyme was doubled, no measurable amylase activity was obtained with the purified amylase preparations but there was evidence of slight amylase activity with the unpurified pancreatin. This slight activity was probably due to small concentrations of chloride or of other favorable ions carried by the pancreatin.

A study of the optimal hydrogen-ion activities and salt concentrations given in Tables I and II, together with the data summarized in Table III, shows that, in order to secure the maximum activation of which each

anion is capable, higher concentrations and more acid solutions are necessary for those anions which are less favorable to the activity of pancreatic amylase than for those which are more efficient activators.

It is evident that misleading values for the relative influence of different anions upon the action of pancreatic amylase will be obtained when comparisons are made at the same hydrogen-ion activity and concentration of anions. Moreover, if a preparation of pancreatic amylase which contains appreciable amounts of chloride is used, the addition of other anions may lead to an apparent inactivation (Table IV). Such considerations undoubtedly explain some of the contradictory statements in the literature concerning the influence of salts on the activity of pancreatic amylase, and probably of other amylases as well.

The ions which favor the activity of pancreatic amylase also increase its stability in aqueous solutions. Pancreatic amylase is much more stable in aqueous solutions containing 0.02 to 0.05 *M* chloride and buffered to *p*H 7.1 with 0.01 *M* phosphate than in distilled water; and this protective influence, noticeable at 5°C, becomes more evident at higher temperatures (Sherman *et al.*, 1910, 1911, 1912, 1926b, 1927a, 1930a; Day, 1934).

Many of the natural amino acids obtained from proteins protect pancreatic amylase from inactivation when its buffered aqueous solutions are held without substrate at 40 or 50°C. These amino acids also increase the activity of pancreatic amylase at these temperatures. Both the stabilizing effect on and the apparent activation of the amylase by these amino acids may be explained in part at least by the protection of the amylase from hydrolysis (Sherman *et al.*, 1912, 1921a,b, 1922a,b, 1923, 1925).

Weidenhagen (1936) reports that 0.001 *M* ascorbic acid causes an inactivation of pancreatic amylase. The unfavorable influence of the ascorbic acid was decreased by the presence of glutathione.

Chemical Nature and Properties. Many investigators have contributed to our knowledge of the purification of pancreatic amylase, and a wide variety of methods has been employed. Outstanding are the fractionation methods of Sherman and Schlesinger (1911, 1912) and the differential adsorption techniques of Willstätter and his co-workers (1923). Combinations of these have also been useful (Sherman *et al.*, 1926a,b, 1930a). Holmbergh (1933b) and Blom *et al.* (1937) have reported that pancreatic amylase, like other dextrinogenic amylases, is readily adsorbed on starches.

The most highly active preparations of pancreatic amylase so far obtained are protein, and the activity is intimately associated with the intact protein molecule (Sherman *et al.*, 1911, 1912, 1915a, 1923, 1926a,b, 1930a, 1931). Although extremely labile, the protein has been crystallized and retains its activity upon recrystallization under carefully controlled

conditions (Caldwell, Booher, and Sherman, 1931). However, the active crystalline protein has so far been obtained in such small yields that it has been used only for activity measurements, studies of crystalline form, and ultimate analyses.

Even when studied under many different conditions and at very different degrees of purification, preparations of pancreatic amylase which have been freed from maltase exhibit remarkably uniform values for the ratio of the saccharogenic to the dextrinogenic activities (Sherman and Schlesinger, 1913b; Holmbergh, 1933b; Orestano, 1933; Day, 1934; Blom, Bak, and Braae, 1937; Little and Caldwell, 1942, 1943). Although measurements of amylase activities are not sufficiently precise to eliminate the possibility of traces of a second amylase, the uniformity of the ratios of the amylase activities strongly suggest that only one amylase is present even in relatively crude preparations of pancreatic amylase.

While the characteristic activity of pancreatic amylase must be considered primarily as a property of the protein molecule as a whole, there is no doubt that it also depends upon the presence and arrangement of the primary amino groups in the molecule. Little and Caldwell (1942, 1943) found that, when these groups were blocked or removed by such reagents as ketene, formaldehyde, or nitrous acid, corresponding decreases in the amylase activities were observed.

Pancreatic amylase differs from pepsin in that the latter was found to retain its proteolytic activity upon the acetylation of its primary amino groups but to lose it upon acetylation of its tyrosine phenolic groups (Herriott and Northrop, 1934; Herriott, 1935).

Exposure to oxidation by bubbling oxygen through solutions of pancreatic amylase did not appreciably influence the amylase activities provided the other conditions were favorable to the stability of the enzyme. No evidence was obtained to indicate that sulfhydryl groups of the protein are essential to the activity of pancreatic amylase (Little and Caldwell, 1943).

SALIVARY AMYLASE

Action on Substrates. Salivary amylase (ptyalin) is a dextrinogenic amylase. It causes a rapid decrease in the viscosity of starch pastes and the rapid hydrolysis of starches to products which give no color with iodine. Köhler-Hollander (1934) reports that the achromic stage is reached when the reducing value is equivalent to 40 to 45 per cent of the theoretical maltose. This corresponds to values of 50 to 55 per cent for pancreatic amylase and to 30 per cent for malt alpha-amylase. The difference in these figures suggests that the hydrolysis of starch is somewhat different in the presence of these three dextrinogenic amylases.

Köhler-Hollander (1934) studied the products formed in the early stages of the hydrolysis of soluble potato starch by salivary amylase. A reaction mixture which gave a red-violet color with iodine and had a reducing value equivalent to 16 per cent of the theoretical maltose was selected. From this mixture he isolated a crystalline reducing dextrin equivalent in weight to 40 to 60 per cent of the original starch. The reducing value and optical rotation indicated a relatively high molecular weight. He concluded that the crystalline material contained a mixture of dextrins made up of 12, 18, and 24 glucose residues in which the 18-unit dextrin predominated.

The crystalline dextrin was hydrolyzed by both salivary and pancreatic amylases to products which had reducing values equivalent to approximately 85 per cent of the theoretical maltose. One hundred per cent hydrolysis of the dextrin is reported for malt alpha- and for malt beta-amylase.

Salivary amylase has been reported to reach a limit in the hydrolysis of starches. According to Vonk and Braak (1934), Köhler-Hollander (1934), and Myrbäck (1943), the limit is reached when the reducing values are equivalent to approximately 80 per cent of the theoretical maltose. Myrbäck (1943) studied the reducing values and the rates of fermentation of reaction mixtures at this stage and concluded that 70 per cent of the reducing value is due to maltose and 30 per cent to "limit dextrins." He fractionated these "dextrins" and reported that 50 per cent were made up of six glucose units, 25 per cent of six to eight glucose units, and 25 per cent of four to six glucose units.

Barbour (1929) and Glock (1936) report that isomaltose is probably an end-product of the action of salivary amylase on glycogen. According to Barbour, the reducing values also indicate the presence of other substances of higher molecular weight; he found no maltose and only occasionally a small amount of glucose.

Chemical Nature. Relatively little is known of the chemical nature of salivary amylase because much of the work with this amylase has been carried out with unpurified saliva or with relatively crude preparations obtained by the dialysis of saliva (Bang, 1911; Michaelis and Pechstein, 1914; Hahn *et al.*, 1920a,b, 1921, 1922; Myrbäck, 1926). Occasionally, the dialysis has been followed by precipitation with alcohol (Cole, 1904).

Ninomiya (1940a) reports that salivary amylase is a globulin. Precipitation occurred with the simultaneous loss of amylase activity during the dialysis of solutions of the amylase against distilled water. The precipitate was redissolved and the activity restored by the addition of neutral salts. Upon fractionation of its solutions with ammonium sulfate, the active amylase was recovered in the "globulin" fractions. Ninomiya has

studied the relationship between the turbidities of solutions of the amylase and their salt concentrations, hydrogen-ion activities, and amylase action. He suggests that the activating influence of salts is due to their ability to dissolve the globulin. Ninomiya (1940b) reports that pepsin acting at *p*H 4.4 completely inactivated salivary amylase; trypsin acting at *p*H 8.0 and papain at *p*H 5.0 had little influence upon the amylase. Tauber and Kleiner (1934) also reported that salivary amylase is slowly inactivated by proteases.

Conditions which Favor the Stability and Activity. Salivary amylase resembles pancreatic amylase in its sensitivity to its chemical environment. This property becomes more pronounced as the amylase is purified (Hahn and Meyer, 1922; the early work is summarized by Adams, 1927). Myrbäck (1926) carried out an extensive investigation of the influence of certain electrolytes on the activities of pancreatic and salivary amylases. H- reports an interrelationship, similar to that already discussed for pancreatie amylase, between the action of salivary amylase and the kind and concenc tration of salt and the hydrogen-ion activities in the reaction mixtures. Further evidence for the similarity of salivary and pancreatic amylases is given by Miller (1931), who reports that the presence or absence of sodium chloride may determine whether or not the thiocyanate ion will exert an apparent activating influence on the action of salivary amylase. This interrelationship was obtained whether the amylase activity was determined by reduction, iodine-color, or viscosity methods. This correlation in data from three types of measurement indicates that a single amylase was present in the salivary amylase preparation used.

Broeze (1928) reports that salivary amylase is inactivated when diluted saliva is held at 60°C for 15 minutes or at 100°C for 5 minutes. The mucin, electrolytes, and other substances present in the saliva undoubtedly protect the amylase under these conditions, and it is probable that highly purified salivary amylase is much more thermolabile than the above data would indicate.

THE AMYLASE OF ASPERGILLUS ORYZAE

The mold *Aspergillus oryzae* is an important commercial source of amylase. Takamine (1898) described a method for the concentration of the amylase and called the product "taka-diastase." This and similar products have found wide use in medicine and industry.

Dextrinogenic amylase predominates in taka-diastase. It causes the rapid hydrolysis of starches to products which give no color with iodine. These exhibit alpha-mutarotation (Kuhn, 1925). Even when studied under different conditions, the ratios of the saccharogenic, dextrinogenic, and

liquefying activities of taka-diastase are remarkably uniform (Blom, Bak, and Braae, 1937; Hollenbeck and Blish, 1941). This correlation in the results obtained with the different types of amylase activity suggests the presence of a single amylase. On the other hand, the above correlation is obtained in the relatively early stages of the amylase action where the presence of maltase and other carbohydrases which accompany the amylase do not appear to influence the results appreciably. Because such enzyme impurities are present in significant concentrations and undoubtedly influence the products formed after the prolonged hydrolysis of starches, it is difficult to evaluate conclusions regarding the mode of action of the amylase when long-continued hydrolyses have been carried out with taka-diastase or similar products.

Purification. The amylase of *Aspergillus oryzae* has been purified by a number of procedures. These include fractionation with alcohol (Sherman and Tanberg, 1916b; Kitano, 1935b), fractionation with salts such as basic lead acetate (Hemmi and Inami, 1929a,b) or ammonium sulfate (Chester, 1933; Werner, 1942), and different adsorption techniques (Nishimura, 1926; Chester, 1933; Kitano, 1936).

The most highly active preparations so far reported are protein. They are maltase free and will be referred to as taka-amylase. They have been obtained from commercially prepared products by repeated fractionation with ammonium sulfate, dialysis, and further fractionation with ethanol (Chester, 1933; Werner, 1942). Dextrinogenic amylase activity predominates throughout the purification, and no evidence is found for the concentration of a saccharogenic amylase component like that obtained in the separation of beta-amylase from extracts of malted barley (Werner, 1942).

Action on Starches. Werner (1942) studied the action of purified maltase-free taka-amylase on soluble potato starch. The selective fermentation method of Somogyi (1937) was used to analyze the products at different stages of the reaction for maltose and glucose. Neither of these sugars could be detected in the early stages of the hydrolysis up to and including reaction mixtures which had reached reducing values equivalent to 18 per cent of the theoretical maltose. When the reducing values reached the equivalent of 25 per cent of the theoretical maltose, which with taka-amylase corresponds to the achromic stage, appreciable concentrations of maltose and possible traces of glucose were present.

Both maltose and glucose were found in significant concentrations in the later stages of the hydrolysis of soluble potato starch by maltase-free taka-amylase. The presence of these sugars was shown by the preferential fermentation of the reaction mixtures by yeast and by the reducing values which continued to rise after 100 per cent of the theoretical yield of

maltose has been attained. The data indicate that the maltose and glucose are formed from the reducing dextrins (Werner, 1942).

Kitano (1935a) studied the fractionation with ethanol of the products of the later stages of the hydrolysis of soluble potato starch by taka-diastase. The material soluble in 94 per cent ethanol was found to contain reducing substances of higher molecular weight than maltose, as evidenced by their yield of glucose upon acid hydrolysis.

Myrbäck (1943) reports that dextrins made up of four and of six glucose units and containing α-1,6- as well as α-1,4-glucosidic linkages are formed in large amounts from a number of different starches by taka-diastase (Ahlborg and Myrbäck, 1938; Myrbäck, Örtenblad, and Ahlborg, 1943b). A study of the products of the hydrolysis of starches by malt beta-amylase, malt alpha-amylase, salivary amylase, and taka-diastase leads Myrbäck (1943) to conclude that α-1,6-glucosidic linkages are not attacked by amylases.

Conditions which Favor the Activity and Stability of Taka-amylase. The activities of taka-amylase, like those of other amylases, are greatly influenced by the conditions under which they are determined. Caldwell and Doebbeling (1937) found that the dextrinogenic (Wohlgemuth) activity of taka-amylase is markedly higher in the presence of sodium chloride than in its absence and that the optimal hydrogen-ion activities depend upon the concentration of sodium chloride in the hydrolysis mixtures. When taka-amylase acts on 1 per cent soluble potato starch for 30 minutes at 40°C in the presence of 0.01 M acetate, the optimal dextrinogenic activity is obtained in the presence of 0.02 to 0.10 M sodium chloride at pH 5.0. Under these conditions, the dextrinogenic activity of purified taka-amylase was approximately twice as high as the maximum activity obtained in the absence of sodium chloride. Unpublished data (Caldwell) indicate that this influence is not due to a specific ion effect.

In measurements of 30 minutes at 40°C with 1 or 2 per cent soluble potato starch and 0.01 M acetate, the presence of sodium chloride appreciably influences the hydrogen-ion activity which is optimal for the saccharogenic activity of taka-amylase, although it does not appreciably influence the saccharogenic activity of this amylase in concentrations up to 0.10 M, provided the hydrogen-ion activities of the reaction mixtures are favorable. The same saccharogenic activity is obtained either at pH 5.0 in the presence of 0.02 to 0.10 M sodium chloride or at pH 5.3 to 5.5 when no sodium chloride is added. Quantitative comparisons of the saccharogenic and dextrinogenic activities of taka-amylase can therefore be made at pH 5.0 in the presence of 0.02 to 0.05 M sodium chloride.

Kitano (1935a) studied the hydrolysis of starch by taka-amylase without the addition of sodium chloride. He also found that, under these

conditions, the saccharogenic activity of taka-amylase is favored by less acid solutions than its dextrinogenic activity. In addition, he reports (1937) that the optimal hydrogen-ion activity for the saccharogenic action of taka-amylase depends upon the temperature and upon the duration of the reaction. Under otherwise similar conditions, less acid solutions were required at a higher temperature (50°C) than at a lower temperature (25°C) and when the time allowed for the hydrolysis of the starch was increased. According to Kitano, the less acid solutions probably favor the stability of the amylase at the higher temperatures and during prolonged hydrolyses.

Ballou and Lück (1940) studied the influence of a number of buffers in systems of a constant ionic strength of 0.05 on the saccharogenic activity of taka-diastase at 30°C. The buffers used were formate, acetate, propionate, butyrate, valerate, phenyl acetate, phthalate, succinate, and citrate. The optimal hydrogen-ion activity was at *p*H 5.1 in the presence of all the buffers studied except phthalate and citrate. With these the optimal hydrogen-ion activity was at *p*H 5.4. The changes of the buffer anions were without influence on the relative activity of the amylase at the optimal hydrogen-ion activity and on the alkaline side of this value; on the acid side, the influence of the buffer ions was quite different.

Hollenbeck and Blish (1941) report that calcium ions protect taka-diastase from heat inactivation.

Amylases are formed not only by *Aspergillus oryzae* but also by a large number of *Aspergilli*, *Penicillia*, and *Mucorales* (Waksman and Davison, 1926). The amylases from the latter sources have not been studied as extensively as that of *Aspergillus oryzae* and will not be discussed here. Bibliographies on the subject are given by Waksman (1922) and by Walton (1928).

Although yeasts have been reported to contain amylases, they are not particularly useful nor important sources of these enzymes. A number of workers have reported that yeasts contain factors which supplement or complement the action of amylases (Pringsheim *et al.*, 1923, 1924, 1926; Nishimura, 1930; Pringsheim *et al.*, 1932). It is probable that these effects are due largely to the action of other enzymes (Weidenhagen and Wolf, 1930; Meyer and Bernfeld, 1942a).

According to Meyer and Bernfeld (1942a), yeasts contain in addition to phosphorylase (see beyond) another enzyme which hydrolyzes starch, glycogen, the residual dextrin formed by beta-amylase, maltose, and even isomaltose. They report that this enzyme is neither alpha-amylase nor beta-amylase; it is not identical with maltase. Meyer and Bernfeld call the enzyme amyloglucosidase and suggest that it may be a mixture of several enzymes.

BACTERIAL AMYLASES

Many amylases of bacterial origin are known which appear to be dextrinogenic amylases similar to those already discussed (Waksman, 1922; Waksman and Davison, 1926; Walton, 1928). However, the information available concerning most of these amylases is meager and does not contribute materially to our present information concerning the chemical nature and mode of action of amylases. Only a few of the bacterial amylases will be referred to here.

The amylases of *Bacillus subtilis* and of *Bacillus mesentericus* have found wide application in industry and have been investigated in considerable detail. Boidin and Effront (1917), who introduced the use of *B. subtilis* for the production of amylase, are credited with making available bacterial amylases for commercial purposes.

The amylase of *B. subtilis* brings about a rapid decrease in the viscosity of starch pastes and hydrolyzes starches rapidly to products which give no color with iodine (Wallerstein, 1939; Hopkins, Dolby, and Stopher, 1942).

Hopkins, Dolby, and Stopher (1942) investigated the products formed from starch by the amylase of *B. subtilis* in hydrolyses carried out at room temperature at *p*H 5.4 and at 75°C at *p*H 7.0. When the hydrolyses at room temperature had reached reducing values equivalent to 33 per cent of the theoretical yield of maltose, the products contained maltose and dextrins of widely varying complexity; at 75°C and at 43 per cent of the theoretical maltose, the more complex dextrins had given place to dextrins of lower molecular weights with relatively little increase in the concentration of maltose. Except for the formation of somewhat larger concentrations of maltose, the products formed by the amylase of *B. subtilis* at room temperature and at *p*H 5.4 were similar to those formed by alpha-amylase from malted barley under the same conditions.

The amylase of *B. subtilis* exerts its optimal activity at *p*H 6.5 to 8.0 (Wallerstein, 1939). It differs from most amylases in its extreme thermostability and exerts marked liquefying activity at temperatures as high as 95°C. In the presence of starch it is possible to boil the reaction mixture for a short period without complete destruction of the liquefying activity (Wallerstein, 1939).

Many commercial bacterial amylase preparations, available under various trade names, have been developed to meet certain industrial needs. These preparations are frequently mixtures of amylases from more than one source and usually contain other carbohydrate-hydrolyzing enzymes as well as amylases. The properties of "Rapidase," "Superclastase,"

and "Biolase" have been described. These amylase preparations are all characterized by high starch-liquefying activities and by their ability to withstand high temperatures.

According to Walton (1928), Rapidase is a preparation obtained from *B. subtilis* or *B. mesentericus* which exerts its activity in slightly acid, neutral, or weakly alkaline solutions, and which acts most rapidly at 70°C.

The properties of Superclastase have been intensively investigated by Lüers and Löther (1935), Lüers and Miller (1935), and Blom, Braae, and Bak (1938). In the early stages of the hydrolyses of starch, when the reducing values are equivalent to 8 per cent of the theoretical maltose, the reducing action of the hydrolysis mixtures of Superclastase is due chiefly to reducing dextrins; after more extensive hydrolysis, maltose as well as reducing dextrins are present.

Biolase has been studied by Pringsheim and Schapiro (1926), Glimm and Gizycki (1932), Janke and Herzog (1937), and Janke, Jirak, and Strunz (1939). It is similar in many respects to Superclastase. Under certain conditions, it has been reported to liberate large amounts of glucose; this property was not attributable to the presence of maltase (Pringsheim and Schapiro, 1926).

Bacillus macerans forms an amylase which has a distinctive action on starches. It hydrolyzes them rapidly to a mixture of water-soluble nonreducing dextrins from which two characteristic nonreducing crystalline compounds may be isolated with ease. These compounds are known as the alpha and beta Schardinger dextrins because they were first reported by Schardinger (1908–09, 1911), who obtained them from starches on which the bacillus had been cultivated. Tilden and Hudson (1939) produced these dextrins from starches by the action of the cell-free sterile amylase. The amylase has been purified and studied by Tilden, Adams, and Hudson (1942), and by McClenahan, Tilden, and Hudson (1942).

The Schardinger dextrins give characteristic colored crystalline compounds with iodine. Those formed by the alpha dextrin are green needles and dark blue hexagons; those formed by the beta dextrin are long yellow needles and orange-brown prisms (Tilden and Hudson, 1942).

Tilden and Hudson (1942) have developed a simple technique for the quantitative measurement of the activity of the amylase of *B. macerans* based upon the use of an iodine test for the crystalline dextrins. The Wohlgemuth type of method may also be used (Adams, unpublished), although the colors with dilute iodine given by the products formed by the *macerans* amylase differ from those obtained with the products formed from starch by any other amylase so far reported.

During the course of the action of the amylase of *B. macerans* on

starches, there is a rapid and marked decrease in the viscosities of the reaction mixtures but no significant increase in the reducing values even after extensive hydrolysis of the starch. The optical activities of the hydrolyzates decrease gradually. The relative proportions of the alpha and beta dextrins formed depend upon the conditions of the reaction. At 20°C the beta dextrin is stable toward the *macerans* amylase, whereas the alpha dextrin is converted, at least in part, to higher rotating material which exhibits slight reducing properties and contains no beta dextrin (McClenahan, Tilden, and Hudson, 1942).

Freudenberg *et al.* (1935, 1936a,b, 1938b) gave evidence that the alpha and beta Schardinger dextrins are closed-ring compounds composed of glucose residues united by α-1,4-glucosidic linkages. They concluded that the alpha dextrin rings contained five glucose units and that the beta dextrin rings contained six glucose units. This conclusion was confirmed by Kratky and Schneidmesser (1938). However, in 1942, French and Rundle presented evidence that the alpha dextrin is a six-glucose ring while the beta dextrin is a seven-glucose ring.

This evidence indicates that the amylase from *B. macerans* causes the cleavage from starch of fragments of six or seven glucose residues in a condition which causes the ends to unite to form closed rings. Yields of the crystalline dextrins amounting to approximately 40 per cent of a starch substrate (Tilden and Hudson, 1939) and to approximately 70 per cent of a crystalline amylose substrate (Kerr, 1942) have been obtained. No evidence exists that these rings are preformed in starches or amyloses in any such concentrations. The alpha and beta Schardinger dextrins therefore offer examples of starch hydrolysis products which were not present as such in the original starches (Kerr, 1942, 1943; Kerr and Severson, 1943; Myrbäck, 1943).

Wilson, Schoch, and Hudson (1943) studied the action of the amylase of *B. macerans* on starches and on their components. They obtained high yields of Schardinger dextrins and minor amounts of noncrystalline "limit" dextrins from the straight-chain components of starches (amyloses). In contrast, they found that the branched-chain components (amylopectins) gave lower yields of Schardinger dextrins and more "limit" dextrins than the original whole starches.

The amylase of *B. macerans* is relatively thermostable. Tilden and Hudson (1939) found no marked inactivation when it was held in aqueous solution at 37.5°C for several days or when it acted on starch at 50°C for one hour. It was relatively stable in the presence of starch even at 70°C. The optimal action of the *macerans* amylase at 40°C was found at hydrogen-ion activities equivalent to *p*H 5.0 to 6.0.

THE PHOSPHORYLASES

The phosphorylases resemble the amylases so far considered in that they also catalyze the degradation of starch and glycogen. They differ from the amylases in a number of important respects. The end-products of the degradation are different, and phosphorylases exhibit marked synthetic action, a property which has not yet been established for amylases. Under suitable conditions and in the presence of inorganic phosphate, phosphorylases catalyze the formation of glucose-1-phosphate from starch or glycogen. If provided with traces of suitable polysaccharide, they also catalyze the reverse reaction or the synthesis of starch-like or glycogen-like material.

Phosphorylases are widely distributed in plant and animal tissues. The occurrence of an enzyme which causes the formation of glucose-1-phosphate from glycogen in the presence of phosphate and adenylic acid was first announced for minced, washed, frog muscle by Cori and Cori (1936, 1937b), and for heart, brain, liver, and yeast, by Cori, Colowick, and Cori (1938a). Hanes (1940a,b,c) reported the presence of phosphorylase in potatoes and in peas. The reversibility of the reaction causing the synthesis of polysaccharide was demonstrated for mammalian phosphorylase by Cori, Schmidt, and Cori (1939a), for yeast phosphorylase by Kiessling (1939b), and for phosphorylase from peas and potatoes by Hanes (1940a,b,c), and has been abundantly confirmed by numerous workers for phosphorylases from different sources.

Phosphorylases have been concentrated and purified by a number of investigators (Cori *et al.*, 1936, 1937a,b, 1938a,b, 1940, 1942, 1943; Kiessling 1939a,b; Schäffner, 1939; Hanes, 1940a,b,c; Green and Stumpf 1941). Purified phosphorylase derived from vertebrate tissues requires the addition of adenylic acid (Cori, 1940, 1941; Cori *et al.*, 1938a,b, 1939b, 1940; Green, Cori, and Cori, 1942), while phosphorylase preparations from plant sources are active in the absence of added adenylic acid (Kiessling, 1939a; Hanes, 1940c; Green and Stumpf, 1941, 1942). These distinctions may be explained by recent findings with muscle phosphorylase (Cori *et al.*, 1942, 1943).

A crystalline adenylic acid complex of phosphorylase was obtained from rabbit muscle by Green, Cori, and Cori (1942). The details of the method are given by Green and Cori (1943). They report that the crystalline enzyme has the characteristics of a globulin. Its solubility in dilute salt solutions is increased by the presence of cysteine. Its molecular weight is reported to lie between 340,000 and 400,000.

Cori and Green (1943) report further that phosphorylase has been obtained from rabbit muscle in two forms: as a crystalline euglobulin which they call phosphorylase *a*, and as a more soluble amorphous protein

which they call phosphorylase *b*. Phosphorylase *a* crystallizes readily and has 60 to 70 per cent of its full activity without the addition of adenylic acid. Phosphorylase *b* is inactive without the addition of adenylic acid and has not yet been crystallized. In the presence of adenylic acid both forms of the enzyme have the same activity per milligram of protein.

Cori and Green (1943) give evidence to show that phosphorylase *a* is a protein complex with a prosthetic group which contains adenylic acid in combination with "an as yet unidentified molecule." Phosphorylase *a* is converted into phosphorylase *b* by the action of an enzyme which splits off the prosthetic group. The latter enzyme accompanies the phosphorylase in the aqueous extracts of rabbit muscle and must be removed early in the purification of the phosphorylase in order to obtain crystalline phosphorylase *a*. The enzyme which splits off the prosthetic group from phosphorylase *a* is also present in heart muscle and in liver and has so far prevented the isolation of crystalline phosphorylase *a* from these sources (1943). The enzyme which splits off the prosthetic group is relatively abundant in spleen.

Phosphorylase *a* is not converted to phosphorylase *b*, that is, the prosthetic group is not split off, by dilution, prolonged dialysis, exposure to extremes of hydrogen-ion activities from *p*H 5.7 to 7.5, or to temperatures which the protein will tolerate without irreversible denaturation. These observations led the authors to conclude that the bond between the enzyme protein and the prosthetic group is not easily dissociated. The nature of this bond has not been elucidated (1943). Although the activity of phosphorylase *a*, lost by the enzymatic removal of the prosthetic group and the formation of phosphorylase *b*, can be fully restored by addition of adenylic acid, the evidence indicates that the addition of adenylic acid to phosphorylase *b* does not result in the formation of an undissociable linkage such as is present in phosphorylase *a*. This firm bond does not appear to be essential to the activity of the phosphorylase.

The activity of plant phosphorylase preparations without the addition of adenylic acid (Kiessling, 1939a; Hanes, 1940c; Green and Stumpf, 1941, 1942) may indicate that these phosphorylases also have a prosthetic group in which adenylic acid is present and that this prosthetic group has not been removed in the purification procedures so far used (December, 1943).

The reactions of phosphorylases have been intensively studied by a number of investigators (Cori and Cori, 1940; Hanes, 1940a,b,c; Hanes and Maskell, 1942; Cori, Cori, and Green, 1943). The work is reviewed by Cori (1940, 1941); by Hanes (1940c); and by Meyer (1943). Further information is also given in Chapter X.

The evidence so far available (Parnas, 1937; Cori, 1940; Meyer,

1943) indicates that the action of phosphorylases in the formation of glucose-1-phosphate from starch and glycogen is analogous to the action of beta-amylase in the formation of maltose from starch. In both cases the action appears to take place at the nonaldehydic ends of the glucose chains. Under the influence of phosphorylase the terminal glucose is esterified with phosphoric acid at carbon 1, with the subsequent breaking off of glucose-1-phosphate; while under the influence of beta-amylase two terminal glucose units are hydrolyzed to form maltose. Thus, as pictured by Parnas (1937) and by Cori (1940), the molecule of phosphoric acid plays a role in "phosphorylysis" by the action of phosphorylases similar to that played by water in the hydrolysis of maltose from starch by the action of beta-amylase.

The synthetic action of phosphorylases is most interesting. Hassid and McCready (1941) obtained evidence by methylation studies that the polysaccharide obtained by the action *in vitro* of purified potato phosphorylase on glucose-1-phosphate consists of long glucopyranose units with little or no branching, and that the synthetic polysaccharide is similar in structure to the amylose fractions of natural starches. This conclusion was confirmed by Bates, French, and Rundle (1943) by iodine titration of the synthetic potato starch obtained by Hassid and McCready (1941). Synthetic potato starch further resembles the amylose component of natural starches in being poorly soluble in water, in giving an intense blue color with iodine, and in being almost completely hydrolyzed by beta-amylase (Hanes, 1940c; Hassid and McCready, 1943). The synthetic polysaccharide obtained by the action of potato phosphorylase on glucose-1-phosphate gave x-ray diffraction patterns similar to those obtained with natural potato starch granules (Astbury, Bell, and Hanes, 1940).

Hassid, Cori, and McCready (1943) report that the polysaccharide synthesized *in vitro* from glucose-1-phosphate by purified muscle phosphorylase is similar in properties to the polysaccharide synthesized *in vitro* by potato phosphorylase. It also resembles the amylose component of starches. It is sparingly soluble in water, retrogrades readily from solution, gives an intense blue color with iodine, is almost completely hydrolyzed to maltose by beta-amylase, and appears from methylation studies to be made up of long unbranched chains of glucopyranose units joined by α-1,4-glucosidic linkages. Bear and Cori (1941) report that the polysaccharide synthesized *in vitro* from glucose-1-phosphate by muscle phosphorylase also gives x-ray diffraction patterns which are very similar to those of natural potato starch.

On the other hand, the polysaccharides synthesized *in vitro* by phosphorylase preparations from yeast (Kiessling, 1939a), or from brain, heart, or liver (Cori and Cori, 1940; Bear and Cori, 1941), resemble glycogen or the branched-chain component of starches. These synthetic polysac-

charides are readily soluble in water, do not show retrogradation, exhibit a reddish brown color with iodine, and give a diffuse x-ray diffraction pattern, characteristic of amorphous material (Bear and Cori, 1941).

Recent work of Cori, Cori, and Green (1943) and of Cori and Cori (1943) explains the apparent difference between the synthetic action of muscle phosphorylase and that of other mammalian phosphorylases, from brain, heart, and muscle. It also explains the finding that muscle phosphorylase brings about the synthesis of a straight-chain polysaccharide (amylose) *in vitro* while it forms a branched-chain polysaccharide (glycogen) *in vivo*. These authors give evidence that an additional enzyme is required to form the α-1,6-glucosidic linkages in the synthetic polysaccharide, and thus to form the branched chains characteristic of glycogen and of the amylopectin components of starches. This supplementary enzyme is removed in the purification of muscle phosphorylase but not in the purification procedures so far developed for the other mammalian phosphorylases. The addition of this supplementary enzyme from heart or liver to purified muscle phosphorylase systems resulted in the synthesis *in vitro* of a polysaccharide which resembled glycogen (Cori and Cori, 1943).

Early work with phosphorylases showed that their synthetic action requires the presence of a trace of a suitable polysaccharide (Cori and Cori, 1939; Hanes, 1940c). According to Cori, Cori, and Green (1943), this requirement shows that synthetic polysaccharide chains are not started under the influence of phosphorylases *in vitro* by a reaction between two glucose-1-phosphate molecules, but indicates that the synthetic process is one of successive additions of glucose-1-phosphate units to existing chains of glucose-1,4-glucosidic linkages. The glucose-1-phosphate molecules are pictured as exchanging their ester bonds for a glucosidic linkage in the chain.

Cori, Cori, and Green (1943) report that the synthetic action of purified muscle phosphorylase *in vitro* requires the addition of glycogen or of the branched-chain component of natural starches, amylopectin. The linear polysaccharides formed by purified muscle or potato phosphorylase *in vitro*, or the linear fraction of natural starch, amylose, cannot be substituted for the glycogen. The authors explain this requirement by pointing out that the linear polysaccharides possess relatively very few end-groups and are poorly soluble in water, while glycogen and amylopectin are highly branched and present many side chains which can be lengthened by the addition of glucose units.

CONCLUSIONS

The importance of amylases and of phosphorylases in nature is attested to by their widespread distribution. Largely due to the out-

standing researches of Cori and Cori and their collaborators, much is already known of the action of phosphorylases and of the part they play in conjunction with other closely related enzymes in the carbohydrate metabolism of the cell (Cori, 1941).

The functions of amylases are still far from clear but it is evident that their action in living tissues is also closely interrelated with that of other enzymes, and that the reactions studied in the laboratory with extracted amylases and more or less isolated systems tell only a part of the story. Thus, maltose and dextrins do not accumulate to any appreciable extent in living tissues although they are important products formed by the action of amylases on starch and glycogen in the laboratory.

On the other hand, exact knowledge concerning the properties and mode of action of the extracted amylases is essential to the intelligent pursuit of information concerning their more complicated interrelationships and functions in the cell. As indicated in this review, much has already been accomplished but much also remains to be done.

The recent advances in our knowledge of the structure and constitution of starches and of glycogen have stimulated renewed interest in amylases and have already suggested new lines of research. They have also illustrated and emphasized the advantages of collaboration between scientific workers of widely different training and points of view. Perhaps the most promising aspect of the amylase problem is the present widespread interest in these enzymes and the resulting impetus to collaborative research by workers in many different fields of scientific and practical endeavor.

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BIBLIOGRAPHY

ADAMS, M. (1927). A quantitative comparison of the influence of neutral salts on the activity of pancreatic amylase and some of the factors involved. *Dissertation*, Columbia University.

—— and HUDSON, C. S. (1943). New methods for the purification of invertase and some properties of the resulting products. *J. Am. Chem. Soc.* **65**:1359–1368.

AHLBORG, K., and MYRBÄCK, K. (1938). Über Grenzdextrine und Stärke. II. Mitteilung: Takadiastase und Maisstärke. *Biochem. Z.* **297**:172–178.

ANDERSON, J. A., and SALLANS, H. R. (1937). Determination of the diastatic power of malt in degrees Lintner by means of a ferricyanide reagent. *Can. J. Research* **C15**:70–77.

ANDERSON, J. A., SALLANS, H. R., and MEREDITH, W. O. S. (1941). Varietal differences in barleys and malts. XII. Summary of correlations between 18 major barley, malt, and malting properties. *Can. J. Research* **C19**:278–291.

ANDREWS, J. S., and BAILEY, C. H. (1934). The amylases of normal and germinated wheat flours. *Cereal Chem.* **11**:551–556.

ASTBURY, W. T., BELL, F. O., and HANES, C. S. (1940). X-ray comparison of natural and synthetic starch. *Nature* **146**:558.

BAILEY, C. H. (1925). *The Chemistry of Wheat Flour.* Chem. Catalog Co., New York.

BAKER, J. L. (1902). The action of ungerminated barley diastase on starch. I. *J. Chem. Soc.* **81**:1177–1185.

—— and HULTON, H. F. E. (1921). Amylases of cereal grains—rye. *J. Chem. Soc.* **119**:805–809.

—— and —— (1922). Amylases of the cereal grains—the "insoluble" amylase of barley. *J. Chem. Soc.* **121**:1929–1934.

—— and —— (1929). Amylases of the cereal grains—oats. *J. Chem. Soc.* **1929**:1655–1660.

BALLOU, G. A., and LUCK, J. M. (1940). Buffer influence on taka-diastase. *J. Biol. Chem.* **135**:111–118.

—— and —— (1941). The effects of different buffers on the activity of β-amylase. *J. Biol. Chem.* **139**:233–240.

BANG, I. (1911). Untersuchungen über Diastasen. I. *Biochem. Z.* **32**:417–442.

BARBOUR, A. D. (1929). Enzymatic hydrolysis of glycogen. *J. Biol. Chem.* **85**: 29–45.

BARKER, C. C., HIRST, E. L., and YOUNG, G. T. (1941). Linkage between the repeating units in the starch molecule. *Nature* **147**:296.

BATES, F. L., FRENCH, D., and RUNDLE, R. E. (1943). Amylose and amylopectin content of starches determined by their iodine complex formation. *J. Am. Chem. Soc.* **65**:142–148.

BEAR, R. S., and CORI, C. F. (1941). X-ray diffraction studies of synthetic polysaccharides. *J. Biol. Chem.* **140**:111–118.

—— and FRENCH, D. (1941). The significance of x-ray diffraction patterns obtained from starch granules. *J. Am. Chem. Soc.* **63**:2298–2305.

BECKMANN, C. O., and LANDIS, Q. (1939a). The molecular weight of beta-amylose from cornstarch by means of the ultracentrifuge. *J. Am. Chem. Soc.* **61**:1495–1503.

—— and —— (1939b). The molecular weight of α-amylo-dextrin (erythro-granulose) from potato starch. *J. Am. Chem. Soc.* **61**:1504–1507.

BERNSTEIN, L. (1943a). Amylases and carbohydrates in developing maize endosperm. *Am. J. Botany* **30**:517–526.

—— (1943b). Hybrid vigor in corn and the mobilization of endosperm reserves. *Am. J. Botany* **30**:801–809.

BLISH, M. J., SANDSTEDT, R. M., and KNEEN, E. (1938). The cereal amylases with reference to flour and malt behavior. *Cereal Chem.* **15**:629–657.

——, ——, and MECHAM, D. K. (1937). Action of wheat amylases on raw wheat starch. *Cereal Chem.* **14**:605–628.

BLOM, J., and BAK, A. (1938). Über die Bestimmung von Amylasen durch Verflüssigung von Stärkekleister. *Z. physiol. Chem.* **256**:197–207.

——, ——, and BRAAE, B. (1936). Untersuchungen über den enzymatischen Abbau der Stärke. *Z. physiol. Chem.* **241**:273–287.

——, ——, and —— (1937). α-Amylasen—β-Amylasen. *Z. physiol. Chem.* **250**:104–122.

——, BRAAE, B., and BAK, A. (1938). Zur Kenntnis der Stärkeabbauprodukte einer α-Amylase. *Z. physiol. Chem.* **252**:261–270.

BOIDIN, A., and EFFRONT, J. (1917). Liquefying amylaceous substances with bacterial enzymes. U. S. Pat. 1,227,374. May 22. (*Chem. Abstracts* **11**:2416.)

BORCHARDT, H., and PRINGSHEIM, H. (1931). Über Malzamylase und Kartoffelamylase. *Biochem. Z.* **239**:193–200.

BOURQUELOT, E. (1887). Sur les caractères de l'affaiblissement éprouvé par la diastase sous l'action de la chaleur. *Compt. rend.* **104**:576–579.

BRIDGMAN, W. B. (1942). Some physical chemical characteristics of glycogen. *J. Am. Chem. Soc.* **64**:2349–2356.

BROEZE, J. R. (1928). Die Einwirkung des Ptyalins auf Stärke. I. *Biochem. Z.* **204**:286–302.

BROWN, H. T., and HERON, J. (1879). Contribution to the history of starch and its transformations. *J. Chem. Soc.* **35**:596–654.

—— and MILLAR, J. H. (1899). Maltodextrin: its oxidation-products and constitution. *J. Chem. Soc.* **75**: 286–308.

—— and MORRIS, G. H. (1885). On the non-crystallisable products of the action of diastase upon starch. *J. Chem. Soc.* **47**:527–570.

—— and —— (1890). Researches on the germination of some of the *Gramineae*. Pt. I. *J. Chem. Soc.* **57**:458–528.

BULBROOK, H. J. (1928). Preparation and properties of the amylase of maize. *Thesis*, Iowa State College.

BURKERT, G. M., and DICKSON, A. D. (1941). The action of the amylases of two barley varieties on the barley starches. *Cereal Chem.* **18**:605–615.

CALDWELL, M. L., BOOHER, L. E., and SHERMAN, H. C. (1931). Crystalline amylase. *Science* **74**:37.

—— and DOEBBELING, S. E. (1935). A study of the concentration and properties of two amylases of barley malt. *J. Biol. Chem.* **110**:739–747.

—— and —— (1937). A quantitative study of the influence of certain factors upon the activity of the amylase of *Aspergillus oryzae*. *J. Am. Chem. Soc.* **59**:1835–1837.

—— and HILDEBRAND, F. C. (1935). A method for the direct and quantitative study of amyloclastic activity of amylases. *J. Biol. Chem.* **111**:411–420.

—— and TYLER, M. G. (1931). A quantitative study of the influence of acetate and of phosphate upon the activity of the amylase of *Aspergillus oryzae*. *J. Am. Chem. Soc.* **53**:2316–2320.

CHESTER, R. M. (1933). A study of the amylase of *Aspergillus oryzae*. *Dissertation*, Columbia University.

CHRZASZCZ, T., and JANICKI, J. (1932). Das Stärkeverflüssigsvermögen der Amylase und seine Bestimmungsmethoden. *Biochem. Z.* **256**:252–291.

—— and —— (1933). Die wirkliche und scheinbare Amylasemenge in verschiedenen Getreidearten vor und während Keimung. *Biochem. Z.* **265**:260–281.

—— and —— (1936). Amylase during the growth and ripening of grains. *Biochem. J.* **30**:1298–1302.

—— and SWIATKOWSKA, W. (1937). Zur Feststellung der wirklichen Amylasemenge im Gerstenschmalz durch Pepton, Proteinasen und Schwefelwasserstoff. *Biochem. Z.* **290**:225–234.

COLE, S. W. (1904). Contributions to our knowledge of the action of enzymes. Part I. The influence of electrolytes on the actions of amylolytic ferments. *J. Physiol.* **30**:202–220.

COLES, J. S. (1941). Ultracentrifugal studies of potato starch, alpha-amylodextrin and their methyl derivatives. *Dissertation*, Columbia University.

CORI, C. F. (1940). Glycogen breakdown and synthesis in animal tissues. *Endocrinology* **26**:285–296.

—— (1941). Phosphorylation of glycogen and glucose. *Biol. Symposia* **5**:131–140.

——, COLOWICK, S. P., and CORI, G. T. (1937a). The isolation and synthesis of glucose-1-phosphoric acid. *J. Biol. Chem.* **121**:465–477.

——, CORI, G. T., and GREEN, A. A. (1943). Crystalline muscle phosphorylase. III. Kinetics. *J. Biol. Chem.* **151**:39–55.

——, SCHMIDT, G., and CORI, G. T. (1939a). The synthesis of a polysaccharide from glucose-1-phosphate in muscle extract. *Science* **89**:464–465.

CORI, G. T., COLOWICK, S. P., and CORI, C. F. (1938a). The formation of glucose-1-phosphoric acid in extracts of mammalian tissues and of yeast. *J. Biol. Chem.* **123**:375–380.

——, ——, and —— (1938b). The action of nucleotides in the disruptive phosphorylation of glycogen. *J. Biol. Chem.* **123**:381–389.

—— and CORI, C. F. (1936). Mechanism of formation of hexose monophosphate in muscle and isolation of a new phosphate ester. *Proc. Soc. Exptl. Biol. Med.* **34**:702–705.

—— and —— (1937b). Formation of glucose-1-phosphoric acid in muscle extract. *Proc. Soc. Exptl. Biol. Med.* **36**:119–122.

—— and —— (1938c). Enzymatic breakdown of glycogen in liver extracts. *Proc. Soc. Exptl. Biol. Med.* **39**:337–338.

—— and —— (1939). The activating effect of glycogen on the enzymatic synthesis of glycogen from glucose-1-phosphate. *J. Biol. Chem.* **131**:397–398.

—— and —— (1940). The kinetics of the enzymatic synthesis of glycogen from glucose-1-phosphate. *J. Biol. Chem.* **135**:733–756.

—— and —— (1943). Crystalline muscle phosphorylase. IV. Formation of glycogen. *J. Biol. Chem.* **151**:57–63.

——, ——, and SCHMIDT, G. (1939b). The role of glucose-1-phosphate in the formation of blood sugar and synthesis of glycogen in the liver. *J. Biol. Chem.* **129**:629–639.

—— and GREEN, A. A. (1943). Crystalline muscle phosphorylase. II. Prosthetic group. *J. Biol. Chem.* **151**:31–38.

CREIGHTON, M., and NAYLOR, N. M. (1933). A study of the starch-digesting and sugar-forming enzymes of wheat. *Iowa State Coll. J. Sci.* **7**:253–260.

DAY, M. H. (1934). Further studies upon the nature and properties of pancreatic amylase. *Dissertation,* Columbia University.

DICKSON, A. D. (1943). A collaborative study of methods for the determination of amylase activity. *Cereal Chem.* **20**:31–35.

—— and BURKHART, B. A. (1942). Changes in the barley kernel during malting: chemical comparisons of germ and distal portions. *Cereal Chem.* **19**:251–262.

DICKSON, J. G., DICKSON, A. D., SHANDS, H. L., and BURKHART, B. A. (1938). Barley and malt studies. IV. Experimental malting of barleys grown in 1936 and summary data for three years 1934–1936. *Cereal Chem.* **15**:133–168.

DÜLL, A. (1935a). Diastase-producing bacteria. *Z. Spiritusind.* **58**:17–18. (*Chem. Abstracts* **29**:6620.)

—— (1935b). Diastatically active yeasts. *Z. Spiritusind.* **58**:31. (*Chem. Abstracts* **29**:5984.)

DULL, M. F., and SWANSON, R. G. (1941). The nature of the liberation of bound barley amylase as effected by salt solutions. *Cereal Chem.* **18**:113–120.

EULER, H. (1922). *Chemie der Enzyme. II. Spezielle Chemie der Enzyme.* Bergmann, Munich.

EULER, H. v., and HELLEBERG, K. (1924). Über die Drehung der bei der Stärkespaltung durch Malzextrakt auftretenden Maltose. *Z. physiol. Chem.* **139**:24–29.

—— and SVANBERG, O. (1920–21). Über die Charakterisierung von Amylaselösungen. *Z. physiol. Chem.* **112**:193–230.

FORD, J. S., and GUTHRIE, J. M. (1908a). Contributions to the biochemistry of barley. I. The amylase of resting barley. *J. Inst. Brewing* **14**:61–87.

—— and —— (1908b). The amylolytic and proteolytic ferments of wheaten flours, and their relations to "baking value." *J. Soc. Chem. Ind.* **27**:389–393.

FOX, T. G. (1943). Ultracentrifugal studies on the amylose and amylopectin components of cornstarch. *Dissertation,* Columbia University.

FREEMAN, G. G., and HOPKINS, R. H. (1936a). The mechanism of degradation of starch by amylases. I. Nature of the early fission products. *Biochem. J* **30**:442–445.

FREEMAN, G. G., and HOPKINS, R. H. (1936b). The mechanism of degradation of starch by amylases. II. Kinetics of action of α- and β-amylases on starch, its components and partial degradation products. *Biochem. J.* **30**:446–450.

—— and —— (1936c). The mechanism of degradation of starch by amylases. III. Mutarotation of fission products. *Biochem. J.* **30**:451–456.

FRENCH, D., and RUNDLE, R. E. (1942). The molecular weights of the Schardinger alpha and beta dextrins. *J. Am. Chem. Soc.* **64**:1651–1653.

FREUDENBERG, K., BLOMQUIST, G., EWALD, L., and SOFF, K. (1936a). Hydrolyse und Acetolyse der Stärke und der Schardinger-Dextrine. *Ber.* **B69**:1258–1266.

—— and BOPPEL, H. (1938a). Methylierte Stärke. *Ber.* **B71**:2505–2511.

—— and —— (1940a). Die Stelle der Verzweigung der Stärkeketten. *Naturwissenschaften* **28**:264.

—— and —— (1940b). Die Lage der Verzweigungstelle in der Stärke. *Ber.* **A73**:609–620.

—— and JACOBI, R. (1935). Über Schardingers Dextrine aus Stärke. *Ann.* **518**:102–108.

—— and MEYER-DELIUS, M. (1938b). Über die Schardinger-Dextrine aus Stärke. *Ber.* **B71**:1596–1600.

—— and RAPP, W. (1936b). Zur Kenntnis der Stärke und der Schardinger-Dextrine. *Ber.* **B69**:2041–2045.

——, SCHAAF, E., DUMPERT, G., and PLOETZ, T. (1939). Neue Ansichten über die Stärke. *Naturwissenschaften* **27**:850–853.

GLIMM, E., and GIZYCKI, I. v. (1932). Zur Kenntnis der Biolase. *Biochem. Z.* **248**:449–473.

GLOCK, G. E. (1936). An investigation of the rates of digestion of starches and glycogen and the bearing on the chemical constitution. I. Action of amylases on starches and glycogen. *Biochem. J.* **30**:1386–1396.

GREEN, A. A., and CORI, G. T. (1943). Crystalline muscle phosphorylase. I. Preparation, properties, and molecular weight. *J. Biol. Chem.* **151**:21–29.

——, ——, and CORI, C. F. (1942). Crystalline muscle phosphorylase. *J. Biol. Chem.* **142**:447–448.

GREEN, D. E., and STUMPF, P. K. (1941). Starch phosphorylase. *J. Biol. Chem.* **140**:xlvii–xlviii (Proc.).

—— and —— (1942). Starch phosphorylase of potato. *J. Biol. Chem.* **142**:355–366.

GRÜTZNER, P. (1876). Notizen über ungeformte Fermente im Säugetierorganismus. *Pflügers Arch. ges. Physiol.* **12**:285–307.

HAHN, A., and HARPUDER, K. (1920a). Über den Einfluss neutraler Alkalisalze auf diastatische Fermente. *Z. Biol.* **71**:287–301.

—— and —— (1920b). Über den Einfluss neutraler Alkalisalze auf diastatische Fermente. *Z. Biol.* **71**:302–310.

—— and MEYER, H. (1922). Über den Einfluss neutraler Alkalisalze auf diastatische Fermente. *Z. Biol.* **76**:227–246.

—— and MICHALIK, R. (1921). Über den Einfluss neutraler Alkalisalze auf diastatische Fermente. *Z. Biol.* **73**:10–18.

HALDANE, J. B. S. (1930). *Enzymes.* pp. 71–73. Longmans, Green, New York.

HANES, C. S. (1932). Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *Biochem. J.* **26**:1406–1421.

—— (1935a). The reversible inhibition of β-malt-amylase by ascorbic acid and related compounds. *Biochem. J.* **29**:2588–2603.

—— (1935b). The action of the two amylases of barley. *Can. J. Research* **B13**:185–208.

—— (1936). The determination of starch in plant tissue, with particular reference to the apple fruit. *Biochem. J.* **30**:168–175.

HANES, C. S. (**1937**). The action of amylases in relation to the structure of starch and its metabolism in the plant. Parts I–III. *New Phytologist* **36**:101–141.

—— (**1940a**). Enzymic synthesis of starch from glucose-1-phosphate. *Nature* **145**:348–349.

—— (**1940b**). The breakdown and synthesis of starch by an enzyme system from pea seeds. *Proc. Roy. Soc. London* **B128**:421–450.

—— (**1940c**). The reversible formation of starch from glucose-1-phosphate catalysed by potato phosphorylase. *Proc. Roy. Soc. London* **B129**:174–208.

—— and CATTLE, M. (**1938**). Starch–iodine coloration as an index of differential degradation by the amylases. *Proc. Roy. Soc. London* **B125**:387–414.

—— and MASKELL, J. E. (**1942**). The influence of hydrogen-ion concentration upon the equilibrium state in phosphorylase systems. *Biochem. J.* **36**:76–79.

HASSID, W. Z., CORI, G. T., and McCREADY, R. M. (**1943**). Constitution of the polysaccharide synthesized by the action of crystalline muscle phosphorylase. *J. Biol. Chem.* **148**:89–96.

—— and McCREADY, R. M. (**1941**). The molecular constitution of enzymically synthesized starch. *J. Am. Chem. Soc.* **63**:2171–2173.

—— and —— (**1943**). The molecular constitution of amylose and amylopectin of potato starch. *J. Am. Chem. Soc.* **65**:1157–1161.

HAWORTH, W. N., HIRST, E. L., and PLANT, M. M. T. (**1935**). Polysaccharides. Part XXI. The constitution and chain-length of some starch dextrins. *J. Chem. Soc.*:1214–1217.

HEMMI, F., and INAMI, G. (**1929a**). Studies on the specific properties of protease and amylase from the standpoint of adsorption phenomena. I. Comparative relation between taka-diastase and pancreatin. *Bull. Agr. Chem. Soc. Japan* **5**:49–54.

—— and —— (**1929b**). Studies on the specific properties of protease and amylase from the standpoint of adsorption phenomena. II. The selective separation of protease and amylase from taka-diastase and pancreatin by means of the method of adsorption. *Bull. Agr. Chem. Soc. Japan* **5**:54–55.

HERRIOTT, R. M. (**1935**). Acetylation of tyrosine in pepsin. *J. Gen. Physiol.* **19**:283–299.

—— and NORTHROP, J. H. (**1934**). Crystalline acetyl derivatives of pepsin. *J. Gen. Physiol.* **18**:35–67.

HILLS, C. H., and BAILEY, C. H. (**1938a**). The nature of the increase in amylase activity of germinating barley. *Cereal Chem.* **15**:273–281.

—— and —— (**1938b**). The amylases of different barley varieties. *Cereal Chem.* **15**:351–362.

HOLLENBECK, C. M., and BLISH, M. J. (**1941**). Parallelism between starch dextrinizing and liquefying activities of amylases. *Cereal Chem.* **18**:754–771.

HOLMBERGH, O. (**1932**). Trennung der zwei Malzamylasen durch Sorption. *Arkiv Kemi Mineral. Geol.* **B11**, No. 4, 6 pp.

—— (**1933a**). Über die Adsorption von α-Amylase aus Malz an Stärke. *Biochem. Z.* **258**:134–140.

—— (**1933b**). Über die Adsorption von α-Amylase. *Biochem. Z.* **266**:203–215.

—— (**1935**). Über die Adsorption von α-Amylase aus Malz an Calziumphosphat. *Arkiv Kemi Mineral. Geol.* **A11**, No. 20, 12 pp.

—— (**1938**). Reinigung von Malzamylase. *Svensk Kem. Tid.* **50**:258–264. (*Chem. Zentr.* **110**:2999–3000. 1939.)

—— (**1940**). Über die Reaktivierung der durch Oxydation inaktivierten Malzamylase. *Svensk Kem. Tid.* 52:107–114. (*Chem. Zentr.* **111**:2623–2630. 1940.)

HOPKINS, R. H., COPE, G. F., and GREEN, J. W. (**1933**). Some observations on barley diastase. *J. Inst. Brewing* **39**:487–493.

——, DOLBY, D. E., and STOPHER, E. G. (**1942**). Some comparisons between a bacterial amylase and malt alpha-amylase. II. The dextrins produced by their action on potato starch. *Wallerstein Lab. Commun.* **5**:125–130.

HOPKINS, R. H., and KULKA, D. (1942). Some comparisons between a bacterial amylase and malt α-amylase. I. The kinetics of starch saccharification by the two enzymes. *J. Inst. Brewing* **48**:170–177. *Wallerstein Lab. Commun.* **5**: 115–124.

—— and ROBERTS, R. H. (1935). The kinetics of alcoholic fermentation of sugars by brewer's yeast. I. Effect of concentrations of yeast and sugar. *Biochem. J.* **29**:919–930.

JANICKI, J. (1939). Oxydo-Reduktionsprozesse bei der Hemmung bezw. Aktivierung der Amylase: Die Rolle der Ascorbinsäure. *Enzymologia* **7**:182–192.

JANKE, A., and HERZOG, F. (1937). Zur Bestimmung des Gerstenextraktes. *Wochschr. Brau.* **54**:233–236.

——, JIRAK, L., and STRUNZ, W. (1939). Die Gerstenextraktbestimmung mittels des Kochmaischbades unter Verwendung von Bakterien-Amylasen. *Wochschr. Brau.* **56**:89–90.

—— and SCHAEFER, B. (1940). Beiträge zur Kenntnis von Stärkeabbau durch Bakterien. *Zentr. Bakt. Parasitenk. II* **102**:241–250.

JÓZSA, S., and GORE, H. C. (1930). Determination of liquefying power of malt diastase. *Ind. Eng. Chem., Anal. Ed.* **2**:26–28.

—— and JOHNSTON, W. R. (1935). Determination of alpha-amylase. *Ind. Eng. Chem., Anal. Ed.* **7**:143–146.

KENDALL, E. C., and SHERMAN, H. C. (1910). Studies on amylases. II. A study of the action of pancreatic amylase. *J. Am. Chem. Soc.* **32**:1087–1105.

KERR, R. W. (1942). Action of *macerans* enzyme on a component of corn starch. *J. Am. Chem. Soc.* **64**:3044–3045.

—— (1943). On the significance of the degradation of starch by *macerans* amylase. *J. Am. Chem. Soc.* **65**:188–193.

—— and SEVERSON, G. M. (1943). On the multiple amylose concept of starch. III. The isolation of an amylose in crystalline form. *J. Am. Chem. Soc.* **65**:193–198.

KIESSLING, W. (1939a). Über den das Glykogen phosphorylierenden Fermentproteinkomplex und eine enzymatische reversible Glykogensynthese. *Biochem. Z.* **302**:50–72.

—— (1939b). Über ein neues Fermentprotein der Hefe und eine reversible enzymatische Synthese des Glykogens. *Naturwissenschaften* **27**:129–130.

KITANO, T. (1935a). Zur Kenntnis der Takaamylase. III. Über die optimale Wasserstoffionenkonzentration bei der Wirkung der Takadiastase. *J. Soc. Chem. Ind. Japan (Suppl.)* **38**:385B–388B.

—— (1935b). Zur Kenntnis der Takaamylase. VI. Über das Verhältnis der amylolytischen und maltatischen Wirksamkeit der Takadiastase bei der Reinigung. *J. Soc. Chem. Ind. Japan (Suppl.)* **38**:449B–450B.

—— (1936). Zur Kenntnis der Takaamylase. XVI. Schlussbetrachtung über die Reinigung der Takaamylase durch Adsorption. *J. Soc. Chem. Ind. Japan* **39**: 391B–392B.

—— (1937). Zur Kenntnis der Takaamylase XVII. Über die Maltasefreie Takaamylase. *J. Soc. Chem. Ind. Japan* **40**:37–38.

KJELDAHL, M. J. (1879–80). Recherches sur les ferments producteurs de sucre. *Compt. rend. trav. lab. Carlsberg* **1**:109–157. Reprinted in *Z. ges. Brauw.* **3**: 49–56, 84–91, 123–128, 145–155, 179–190, 222–225 (1880).

VAN KLINKENBERG, G. A. (1932a). Über die Spezifität der Amylasen. I. Trennung und Eigenschaften der beiden Malzamylasen. *Z. physiol. Chem.* **209**: 253–268.

—— (1932b). Über die Spezifität der Amylasen. II. Die enzymatische Analyse von Stärke und Glykogen. *Z. physiol. Chem.* **212**:173–195.

—— (1934). Das Spezifitätsproblem der Amylasen. *Ergeb. Enzymforsch.* **3**:73–94.

KNEEN, E. (1943). The cereal amylases. Significance of the component concept. *Wallerstein Lab. Commun.* **6**:101–110.

—— (1944). A comparative study of the development of amylases in germinating cereals. *Cereal Chem.* **21**:304–314.

KNEEN, E., BECKORD, O. C., and SANDSTEDT, R. M. (1941a). Starch degrading properties of barley malts. *Cereal Chem.* **18**:741–754.

——, MILLER, B. S., and SANDSTEDT, R. M. (1942). The influence of temperature in the development of amylase in germinating wheat. *Cereal Chem.* **19**:11–27.

—— and SANDSTEDT, R. M. (1941b). Beta-amylase activity and its determination in germinated and ungerminated cereals. *Cereal Chem.* **18**:237–252.

—— and —— (1943a). An amylase inhibitor from certain cereals. *J. Am. Chem. Soc.* **65**:1247.

——, ——, and HOLLENBECK, C. M. (1943b). The differential stability of the malt amylases—separation of the alpha and beta components. *Cereal Chem.* **20**: 399–423.

KÖHLER-HOLLANDER, L. (1934). Über krystallisiertes Erythrodextrin. *Z. physiol. Chem.* **228**:249–255.

KRATKY, O., and SCHNEIDMESSER, B. (1938). Die röntgenographische Untersuchung des Schardingerschen α-Dextrins. *Ber.* **B71**:1413–1414.

KUHN, R. (1924). Über die Konstitution der Stärke und die verschiedenen Wirkungsweisen der Amylasen. *Ber.* **57**:1965–1968.

—— (1925). Der Wirkungsmechanismus der Amylasen; ein Beitrag zum Konfigurationsproblem der Stärke. *Ann.* **443**:1–71.

LAURENS, H. (1933). *The Physiological Effects of Radiant Energy.* p. 565. Chem. Catalog Co., New York.

LINDERSTRØM-LANG, K., and ENGEL, C. (1937). Über die Verteilung der Amylase in den ausseren Schichten des Gerstenkornes. *Enzymologia* **3**: 138–146.

LING, A. R., and BAKER, J. L. (1897). Action of diastase on starch. *J. Chem. Soc.* **71**:508–522.

—— and CARTER, W. A. (1938). Studies on starch. A polyhexosan (the so-call stable dextrin of H. T. Brown). *J. Inst. Brewing* **44**:424.

——, ——, and POTTER, R. S. (1938). Studies on starch. A polyamylose, Baker's α-amylodextrin. *J. Inst. Brewing* **44**:422–423.

—— and NANJI, D. R. (1923). Studies on starch. Part I. The nature of polymerized amylose and amylopectin. *J. Chem. Soc.* **123**:2666–2688.

—— and —— (1925). Studies on starch. Part III. The nature and the genesis of the stable dextrin and of the maltodextrins. *J. Chem. Soc.* **127**:636–651.

LINTNER, C. J. (1886). Studien über Diastase. I. *J. prakt. Chem.* **34**:378–394.

—— (1887). Studien über Diastase. II. *J. prakt. Chem.* **36**:481–498.

—— and ECKHARDT, F. (1890). Studien über Diastase. III. *J. prakt. Chem.* **41**: 91–96.

LITTLE, J. E., and CALDWELL, M. L. (1942). A study of the action of pancreatic amylase. *J. Biol. Chem.* **142**:585–595.

—— and —— (1943). A study of the action of pancreatic amylase. II. *J. Biol. Chem.* **147**:229–232.

LÜERS, H. (1935). Die Entwicklung der Enzyme beim Mälzen. *Wochschr. Brau.* **52**:249–254.

—— (1936). Changes in enzymes during malting. *Cereal Chem.* **13**:153–171.

—— and LECHNER, R. (1933). Einwirkung von Papain auf Gerste und Malz und deren Auszüge. *Wochschr. Brau.* **50**:33–38.

—— and LÖTHER, A. (1935). Die viskosimetrische Verfolgung von Enzymreaktionen. *Wochschr. Brau.* **52**:49–51.

—— and MILLER, P. (1935). Eine neue Gerstenextrakt Bestimmungsmethode. *Wochschr. Brau.* **52**:153–154.

—— and RÜMMLER, W. (1933). Die Entwicklung der Amylase während der Keimung der Gerste. *Wochschr. Brau.* **50**:297–300.

—— and —— (1935). Die Entwicklung der Amylasen während der Keimung der Gerste. *Wochschr. Brau.* **52**:9–12.

—— and SELLNER, E. (1925). Beiträge zur Reinigung der Malzamylase. *Wochschr. Brau.* **42**:97–99.

LUTZ, J. G., and NELSON, J. M. (**1934**). Preparation of highly active yeast invertase. *J. Biol. Chem.* **107**:169–177.

McCLENAHAN, W. S., TILDEN, E. B., and HUDSON, C. S. (**1942**). A study of the products obtained from starch by the action of the amylase of *Bacillus macerans*. *J. Am. Chem. Soc.* **64**:2139–2144.

McCREADY, R. M., and HASSID, W. Z. (**1943**). The separation and quantitative estimation of amylose and amylopectin in potato starch. *J. Am. Chem. Soc.* **65**:1154–1157.

MÄRKER, M. (**1878**). Standpunkt unserer Kenntniss über die diastatischen Vorgänge. *Chem. Zentr. III* **9**:559–560.

MARTIN, V. D., NAYLOR, N. M., and HIXON, R. M. (**1939**). Action of beta-amylase from soybeans on various starches. *Cereal Chem.* **16**:565–573.

MAYER, K., and KLINGA-MAYER, M. (**1940**). Über das Stärkeverflüssigungsvermögen während der Keimung der Gerste. *Z. physiol. Chem.* **267**:115–127.

MEYER, K. H. (**1942**). Recent developments in starch chemistry. In *Advances in Colloid Science*, Vol. I, pp. 143–182. Interscience, New York. See also a series of papers published in *Helv. Chim. Acta* summarized by Meyer, 1942, 1943.

—— (**1943**). The chemistry of glycogen. In *Advances in Enzymology*, Vol. III, pp. 109–135. Interscience, New York.

—— and BERNFELD, P. (**1940a**). Recherches sur l'amidon V. L'amylopectine. *Helv. Chim. Acta* **23**:875–885.

—— and —— (**1941a**). Recherches sur l'amidon XIV. La réaction colorée à l'iode de l'amidon et du glycogène. *Helv. Chim. Acta* **24**:389–393.

—— and —— (**1942a**). Recherches sur l'amidon XXI. Sur les enzymes amylolytiques de la levure. *Helv. Chim. Acta* **25**:399–403.

—— and —— (**1942b**). Recherches sur l'amidon XXII. L'action de la phosphorylase de pommes de terre. *Helv. Chim. Acta* **25**:404–405.

——, ——, and PRESS, J. (**1940b**). Recherches sur l'amidon VIII. La dégradation des constituants de l'amidon par la β-amylase. *Helv. Chim. Acta* **23**:1465–1476.

——, ——, and WOLFF, E. (**1940c**). Recherches sur l'amidon III. Fractionnement et purification de l'amylose de maïs naturel. *Helv. Chim. Acta* **23**:854–864.

——, BRENTANO, W., and BERNFELD, P. (**1940d**). Recherches sur l'amidon II. Sur la nonhomogénéité de l'amidon. *Helv. Chim. Acta* **23**:845–853.

—— and FULD, M. (**1941b**). Recherches sur l'amidon XII. L'arrangement des restes de glucose dans le glycogène. *Helv. Chim. Acta* **24**:375–378.

—— and PRESS, J. (**1941c**). Recherches sur l'amidon X. La dégradation du glycogène par la β-amylase. *Helv. Chim. Acta* **24**:58–62.

——, WERTHEIM, M., and BERNFELD, P. (**1940e**). Recherches sur l'amidon IV. Méthylation et determination des groupes terminaux d'amylose et d'amylopectine de maïs. *Helv. Chim. Acta* **23**:865–875.

——, ——, and —— (**1941d**). Recherches sur l'amidon XI. Sur la dextrine residuelle de l'amidon de maïs (erythrogranulose). *Helv. Chim. Acta* **24**:212–216.

——, ——, and —— (**1941e**). Recherches sur l'amidon XIII. Contribution a l'étude de l'amidon de pommes de terre. *Helv. Chim. Acta* **24**: 378–389.

MICHAELIS, L., and PECHSTEIN, H. (**1914**). Die Wirkungsbedingungen der Speicheldiastase. *Biochem. Z.* **59**:77–99.

MILLER, L. P. (**1931**). The effect of thiocyanates upon amylase activity. II. Salivary amylase. *Contrib. Boyce Thompson Inst.* **3**:287–296.

MYRBÄCK, K. (**1926**). Über Verbindungen einiger Enzyme mit inaktivierenden Stoffen. II. *Z. physiol. Chem.* **159**:1–84.

—— (**1936**). Zur Kenntnis der Stärkespaltung durch pflanzliche Amylasen. I. *Biochem. Z.* **285**:290–293.

—— (**1938a**). Über Grenzdextrine und Stärke. I. Mitteilung. *Biochem. Z.* **297**: 160–171.

MYRBÄCK, K. (**1938b**). Über Grenzdextrine und Stärke. III. Mitteilung: Trisaccharide unter den Abbauprodukten der Stärke. *Biochem. Z.* **297**:179–183.

—— (**1941a**). Über Grenzdextrine und Stärke. X. Primärprodukte der Stärkespaltung durch Dextrinogenamylase. *Biochem. Z.* **307**:132–139.

—— (**1941b**). Über Grenzdextrine und Stärke. XI. Spaltung der Stärke durch die Dextrinogenamylase des Malzes. *Biochem. Z.* **307**:140–158.

—— (**1943**). Der enzymatische Abbau der Stärke und der Bau der Stärkemakromoleküle. *J. prakt. Chem.* **162**:29–62.

—— and AHLBORG, K. (**1940a**). Über Grenzdextrine und Stärke. VIII. Die Konstitution eines Stärkegrenzdextrins. Nachweis α-glykosidischer 1,6-Bindungen in Dextrin und Stärke. *Biochem. Z.* **307**:69–78.

—— and KIHLBERG, B. (**1943a**). Über Grenzdextrine und Stärke. XIX. Über die Phosphorsäure in Stärkedextrinen. *Biochem. Z.* **315**:250–258.

—— and MYRBÄCK, S. (**1933**). Über Gersten- und Malzamylase. *Biochem. Z.* **258**:158–171.

—— and —— (**1936**). Über freie und gebundene Amylase in der Gerste. *Biochem. Z.* **285**:282–289.

—— and ÖRTENBLAD, B. (**1937–38**). Über aktive und inaktive Amylase in Gerste. *Enzymologia* **2**:305–309.

——, ——, and AHLBORG, K. (**1940b**). Über Grenzdextrine und Stärke. VI. Grenzdextrine aus Kartoffelstärke mittels Pankreasamylase. *Biochem. Z.* **307**:49–52.

——, ——, and —— (**1940c**). Über Grenzdextrine und Stärke. VII. Schwerhydrolysierbare Glykosidbindungen in der Stärke. *Biochem. Z.* **307**:53–68.

——, ——, and —— (**1943b**). Über Grenzdextrine und Stärke. XVIII. Über Grenzdextrine aus Kartoffel-, Reis- und Weizenstärke. *Biochem. Z.* **315**:240–249.

NAYLOR, N. M., SPENCER, M., and HOUSE, M. (**1925**). The preparation and properties of amylase from germinated wheat and rye. *J. Am. Chem. Soc.* **47**:3037–3039.

NELSON, D. H., and DICKSON, A. D. (**1942**). The application of the Kneen and Sandstedt method for α- and β-amylase to malts from different barley varieties. *Am. Soc. Brewing Chem., Proc. 5th Ann. Meeting*:61–67.

NEWTON, J. M., HIXON, R. M., and NAYLOR, N. M. (**1943**). Soybean amylase. II. Further purification of crude soybean beta-amylase concentrates. *Cereal Chem.* **20**:23–31.

—— and NAYLOR, N. M. (**1939**). Soybean amylase. I. The concentration and characterization of soybean amylase. *Cereal Chem.* **16**:71–78.

NINOMIYA, H. (**1940a**). Beitrag zur Kenntnis der Speichelamylase. I. Mitteilung: Über die Globulinnatur der Speichelamylase. *J. Biochem. Japan* **31**:69–78.

—— (**1940b**). Beitrag zur Kenntnis der Speichelamylase. II. Mitteilung: Über die Spaltung der Speichelamylase durch Proteasan und über ihren Kohlenhydrat- und Eisengehalt. *J. Biochem. Japan* **31**:421–428.

NISHIMURA, S. (**1926**). Über die Reinigung der Amylase aus *Aspergillus Oryzae*. *Bull. Agr. Chem. Soc. Japan* **2**:129–146.

—— (**1930**). Über das Stärke verflüssigende Enzym in Trochenhefe-autolysaten (Synthetische Wirkungen des Enzyms). *Biochem. Z.* **223**:161–170.

NORDH, G., and OHLSSON, E. (**1932**). Amylasen in ruhenden und keimenden Samen. I. Gerste. *Z. physiol. Chem.* **204**:89–100.

NORTHROP, J. H. (**1934–35**). *Harvey Lectures, Ser.* **30**:229–270.

—— (**1937**). Chemical nature and mode of formation of pepsin, trypsin and bacteriophage. *Science* **86**:479–483, 487.

ÖRTENBLAD, B., and MYRBÄCK, K. (**1941a**). Über Grenzdextrine und Stärke. IX. Primäre Spaltung der Stärke durch die Dextrinogenamylase des Malzes. *Biochem. Z.* **307**:123–128.

—— and —— (**1941b**). Amylasewirkung auf oxydierte Stärke. *Biochem. Z.* **307**:129–131.

ÖRTENBLAD, B., and MYRBÄCK, K. (1943). Über Grenzdextrine und Stärke. XVII. Über den Mechanismus der Spaltung durch Dextrinogenamylase. *Biochem. Z.* **315**:233–239.

OHLSON, W. J., BURKHART, B. A., and DICKSON, A. D. (1943). Inactivation of alpha- and beta-amylase in aqueous malt extracts. *Cereal Chem.* **20**:126–138.

OHLSSON, E. (1922). Sur l'existence de deux ferments amylolytiques dans la diastase du malt. *Compt. rend. soc. biol.* **87**:1183–1184.

—— (1926). The two components of malt diastase. *Compt. rend. trav. lab. Carlsberg* **16**, No. 7: 1–68.

—— (1930). Über die beiden Komponenten der Malzdiastase, besonders mit Rücksicht auf die Mutarotation der bei der Hydrolyse der Stärke gebildeten Produkte. *Z. physiol. Chem.* **189**:17–63.

—— and EDFELDT, O. (1933). Amylasen in ruhenden und keimenden Samen. III. Hafer. *Z. physiol. Chem.* **221**:174–179.

—— and THÖRN, N. (1938). Amylasen in ruhenden und keimenden Samen. V: Die Entwicklung der Alpha-Amylase der keimenden Gerste. *Compt. rend. trav. lab. Carlsberg, Sér. Chim.* **22**:398–404.

—— and UDDENBERG, C. E. (1933). Amylasen in ruhenden und keimenden Samen. II. Roggen. *Z. physiol. Chem.* **221**:165–173.

ORESTANO, G. (1933). The liquefaction and saccharification of starch by the action of amylases from different sources. *Arch. farmacol. sper.* **56**:383–406. (*Chem. Abstracts* **28**:1721).

OSBORNE, T. B. (1895). The chemical nature of diastase. *J. Am. Chem. Soc.* **17**: 587–603.

—— and CAMPBELL, G. F. (1896). The chemical nature of diastase. *J. Am. Chem. Soc.* **18**:536–542.

O'SULLIVAN, C. (1876). On the action of malt-extract on starch. *J. Chem. Soc.* **30**: 125–144.

—— (1879). On the transformation-products of starch. *J. Chem. Soc.* **35**:770–785.

PARNAS, J. K. (1937). Der Mechanismus der Glycogenolyse im Muskel. *Ergeb. Enzymforsch.* **6**:57–110.

PRINGSHEIM, H., and BEISER, A. (1924). Über ein Komplement der Amylasen und das Grenzdextrin. *Biochem. Z.* **148**:336–343.

——, BORCHARDT, H., and HUPFER, H. (1932). Über Glutathion als Komplement der Amylasen. *Biochem. Z.* **250**:109–124.

—— and GINSBURG, S. (1935). L'amylolyse et l'ester phosphorique de l'amidon et du glycogène. *Bull. soc. chim. biol.* **17**:1599–1606.

—— and LEIBOWITZ, J. (1926). Über die Spezifität der Amylasen. *Ber.* **59**:991–995.

—— and OTTO, G. (1926). Über das Komplement der Amylasen. IV. *Biochem. Z.* **173**:399–410.

—— and SCHAPIRO, E. (1926). Über den fermentativen Abbau der Stärke durch "Biolase." *Ber.* **59**:996–1000.

— and SCHMALZ, K. (1923). Über den Grenzabbau der Stärke und ein Komplement der Amylasen. *Biochem. Z.* **142**:108–116.

PRIOR, E., and WIEGMANN, D. (1900). Darstellung und Eigenschaften des Diastase-Achroodextrins. III. *Z. angew. Chem.* **13**:464–469.

PRONIN, S. (1931). Einfluss der Temperatur auf die Bildung reduzierender Stoffe bei der Selbstverzuckerung des Roggenmehls. *Biochem. Z.* **240**:94–97.

PURR, A. (1934). The influence of vitamin C (ascorbic acid) on plant and animal amylases. *Biochem. J.* **28**:1141–1148.

ROBERTS, W. (1881). Estimation of the amylolytic and proteolytic activity of pancreatic extracts. *Proc. Roy. Soc. London* **32**:145–161. *J. Chem. Soc. Abs.* **40**: 1051–1054.

RONA, P., and VAN EWEYK, C. (1924). Eine neue Methode zur Untersuchung der Amylase. *Biochem. Z.* **149**:174–187.

RUNDLE, R. E., and BALDWIN, R. R. (1943). The configuration of starch and the starch–iodine complex. I. The dichroism of flow of starch–iodine solutions. *J. Am. Chem. Soc.* **65**:554–558.

RUNDLE, R. E., and FRENCH, D. (1943). The configuration of starch and the starch–iodine complex. II. Optical properties of crystalline starch fractions. *J. Am. Chem. Soc.* **65**:558–561.

SAMEC, M. (1935). Über die Wirkung von β-amylase auf einige Stärkesubstanzen. *Z. physiol. Chem.* **236**:103–118.

—— (1937). Über die Wirkung von α-Malzamylase auf einige Stärkesubstanzen. *Z. physiol. Chem.* **248**:117–128.

—— (1939). Die Anfangsphase der α-diastatischen Stärkehydrolyse. *Z. physiol. Chem.* **259**:204–212.

—— (1940). Die neueren Ergebnisse der Stärkeforschung. III. Die Konstitution der Stärke. *Kolloid-Beihefte* **51**:359–492.

—— and BLINC, M. (1938). Die neueren Ergebnisse der Stärkeforschung. Die Bestandteile des Stärkekornes I. Fraktionierung der Kornbestandteile. *Kolloid-Beihefte* **47**:371–472.

—— and —— (1939). Die neueren Ergebnisse der Stärkeforschung II. Der Abbau der Stärke. *Kolloid-Beihefte* **49**:75–314.

—— and WALDSCHMIDT-LEITZ, E. (1931). Über die enzymatische Spaltbarkeit der Amylo- und Erythrokörper aus Stärke. *Z. physiol. Chem.* **203**:16–33.

SANDSTEDT, R. M., BLISH, M. J., MECHAM, D. K., and BODE, C. E. (1937). Identification and measurement of factors governing diastasis in wheat flour. *Cereal Chem.* **14**:17–34.

——, KNEEN, E., and BLISH, M. J. (1939). A standardized Wohlgemuth procedure for alpha-amylase activity. *Cereal Chem.* **16**:712–723.

SCHÄFFNER, A. (1939). Die enzymatische Synthese des Glykogens. *Naturwissenschaften* **27**:195.

SCHARDINGER, F. (1908–09). Über die Bildung krystallisierter Fehlingsche Lösung nicht reduzierender Körper (Polysaccharide) aus Stärke durch mikrobielle Tätigkeit. *Zentr. Bakt. Parasitenk. II* **22**:98–103.

—— (1911). Bildung krystallisierter Polysaccharide (Dextrine) aus Stärkekleister durch Mikrobin. *Zentr. Bakt. Parasitenk. II* **29**:188–197.

SCHOCH, T. J. (1942). Fractionation of starch by selective precipitation with butanol. *J. Am. Chem. Soc.* **64**:2957–2961.

SHERMAN, H. C. (1923). An investigation of the chemical nature of two typical enzymes: Pancreatic and malt amylases. *Proc. Natl. Acad. Sci. U. S.* **9**:81–86.

—— and CALDWELL, M. L. (1921a). A study of the influence of arginine, histidine, tryptophane and cystine upon the hydrolysis of starch by purified pancreatic amylase. *J. Am. Chem. Soc.* **43**:2469–2476.

—— and —— (1922a). Influence of lysine upon the hydrolysis of starch by purified pancreatic amylase. *J. Am. Chem. Soc.* **44**:2926–2930.

——, ——, and ADAMS, M. (1926a). Further experiments upon the purification of pancreatic amylase. *Proc. Soc. Exptl. Biol. Med.* **23**:413–416.

——, ——, and —— (1926b). Enzyme purification by adsorption: An investigation of pancreatic amylase. *J. Am. Chem. Soc.* **48**:2947–2956.

——, ——, and —— (1927a). Establishment of the optimal hydrogen-ion activities for the enzymic hydrolysis of starch by pancreatic and malt amylases under varied conditions of time and temperature. *J. Am. Chem. Soc.* **49**:2000–2005.

——, ——, and —— (1928a). The interrelation of hydrogen-ion activity and concentration of salt in the activation of pancreatic amylase. *J. Am. Chem. Soc.* **50**:2529–2535.

——, ——, and —— (1928b). The influence of concentration of neutral salt on the activation of pancreatic amylase. *J. Am. Chem. Soc.* **50**:2535–2537.

——, ——, and —— (1928c). A quantitative comparison of the influence of neutral salts on the activity of pancreatic amylase. *J. Am. Chem. Soc.* **50**:2538–2543.

——, ——, and —— (1930a). Enzyme purification: further experiments with pancreatic amylase. *J. Biol. Chem.* **88**:295–304.

SHERMAN, H. C., CALDWELL, M. L., and BOYNTON, H. H. (**1930b**). A quantitative study of the influence of acetate and of phosphate on the activity of malt amylase. *J. Am. Chem. Soc.* **52**:1669–1672.

——, ——, and CLEAVELAND, M. (**1930c**). The influence of certain neutral salts upon the activity of malt amylase. *J. Am. Chem. Soc.* **52**:2436–2440.

——, ——, and DALE, J. E. (**1927b**). A quantitative study of the influence of sodium acetate, sodium borate, sodium citrate and sodium phosphate upon the activity of pancreatic amylase. *J. Am. Chem. Soc.* **49**:2596–2598.

——, ——, and DOEBBELING, S. E. (**1934**). Further studies upon the purification and properties of malt amylase. *J. Biol. Chem.* **104**:501–509.

——, ——, and NAYLOR, N. M. (**1925**). Influence of tryptophan and other amino acids upon the stability and enzymic activity of pancreatic amylase. *J. Am. Chem. Soc.* **47**:1702–1709.

—— and GETTLER, A. O. (**1913a**). Studies on amylases. VII. The forms of nitrogen in amylase preparations from the pancreas and from malt, as shown by the Van Slyke method. *J. Am. Chem. Soc.* **35**:1790–1794.

——, KENDALL, E. C., and CLARK, E. D. (**1910**). Studies on amylases. I. An examination of methods for the determination of diastatic power. *J. Am. Chem. Soc.* **32**:1073–1086.

—— and NAYLOR, N. M. (**1922b**). Influence of some organic compounds upon the hydrolysis of starch by salivary and pancreatic amylases. *J. Am. Chem. Soc.* **44**:2957–2966.

—— and PUNNETT, P. W. (**1916a**). On the products of the action of certain amylases upon soluble starch, with special reference to the formation of glucose. *J. Am. Chem. Soc.* **38**:1877–1885.

—— and SCHLESINGER, M. D. (**1911**). Studies on amylases. III. Experiments upon the preparation and properties of pancreatic amylase. *J. Am. Chem. Soc.* **33**:1195–1204.

—— and —— (**1912**). Studies on amylases. IV. A further investigation of the properties of pancreatic amylase. *J. Am. Chem. Soc.* **34**:1104–1111.

—— and —— (**1913b**). Studies on amylases. VI. A comparison of amyloclastic and saccharogenic powers. *J. Am. Chem. Soc.* **35**:1784–1790.

—— and —— (**1915a**). Studies on amylases. IX. Further experiments upon the purification of malt amylase. *J. Am. Chem. Soc.* **37**:643–648.

—— and TANBERG, A. P. (**1916b**). Experiments upon the amylase of *Aspergillus oryzae*. *J. Am. Chem. Soc.* **38**:1638–1645.

—— and THOMAS, A. W. (**1915b**). Studies on amylases. VIII. The influence of certain acids and salts upon the activity of malt amylase. *J. Am. Chem. Soc.* **37**:623–643.

—— and WALKER, F. (**1921b**). The influence of certain amino acids upon the enzymic hydrolysis of starch. *J. Am. Chem. Soc.* **43**:2461–2469.

—— and —— (**1923**). Effect of amino acids in retarding the hydrolytic decomposition of an enzyme (pancreatic amylase). *J. Am. Chem. Soc.* **45**:1960–1964.

SIZER, I. W. (**1943**). Effects of temperature on enzyme kinetics. In *Advances in Enzymology*, Vol. III, pp. 35–62. Interscience, New York.

SJÖBERG, K. (**1923**). Über ein Amylasepräparat mit beschränktem Spaltungsvermögen. *Z. physiol. Chem.* **131**:116–130.

—— and ERIKSSON, E. (**1924**). Über Amylase. *Z. physiol. Chem.* **139**:118–139.

SMALL, J. C. (**1919**). A method for the preparation of soluble starch. *J. Am. Chem. Soc.* **41**:113–120.

SNIDER, S. R. (**1940**). The determination of total β-amylase in barley and barley malt. *Am. Soc. Brewing Chem., Proc. 3rd Ann. Meeting*:49–62.

SOMOGYI, M. (**1937**). Detection and quantitative determination of small amounts of glucose in mixtures containing maltose. *J. Biol. Chem.* **119**:741–747.

STAMBERG, O. E., and BAILEY, C. H. (**1938**). Action of wheat amylases on soluble starch. *J. Biol. Chem.* **126**:479–488.

STAMBERG, O. E., and BAILEY, C. H. (**1939**). Studies on wheat starch. I. The amylopectin and amylose content of various wheat starches. *Cereal Chem.* **16**:309–319.

SUMNER, J. B. (**1933**). The chemical nature of enzymes. *J. Nutrition* **6**:103–112.

SYNIEWSKI, V. (**1902**). Ueber die Constitution der Stärke. *Ann.* **324**:212–268.

—— (**1925**). Über nichtreduzierendes Grenzdextrin. I. *Ann.* **441**:285–297.

TAKAMINE, J. (**1898**). Diastatic substances from fungus growths. *J. Soc. Chem. Ind.* **17**:118–120.

TAUBER, H., and KLEINER, I. S. (**1934**). The inactivation of pepsin, trypsin, and salivary amylase by proteases. *J. Biol. Chem.* **105**:411–414.

TELLER, G. L. (**1936**). Evidence concerning two types of plant diastase. *J. Biol. Chem.* **114**:425–430.

TILDEN, E. B., ADAMS, M., and HUDSON, C. S. (**1942**). Purification of the amylase of *Bacillus macerans*. *J. Am. Chem. Soc.* **64**:1432–1433.

—— and HUDSON, C. S. (**1939**). The conversion of starch to crystalline dextrins by the action of a new type of amylase separated from cultures of *Aero-bacillus macerans*. *J. Am. Chem. Soc.* **61**:2900–2902.

—— and —— (**1942**). Preparation and properties of the amylases produced by *Bacillus macerans* and *Bacillus polymyxa*. *J. Bact.* **43**:527–544.

TYCHOWSKI, A. (**1937**). Untersuchungen über die Stärke. Grenzabbau von Stärken verschiedener Herkunft bei Einwirkung verschiedener Getreideamylasen und Untersuchung der Hydrolyseprodukte. *Biochem. Z.* **291**:138–158.

VENKATA-GIRI, K. (**1934**). Amylase from sweet potato (*Ipomoea batatas*). *J. Indian Chem. Soc.* **11**:339–350.

—— and SREENIVASAN, A. (**1937**). Das Amylasesystem des Reiskornes während des Reifens und Keimens. *Biochem. Z.* **289**:155–166.

VONK, H. J., and BRAAK, J. P. (**1934**). Die Abbaugrenzen der Stärke bei der Einwirkung von Speichel- und Pankreasamylase. *Proc. Acad. Sci. Amsterdam* **37**:188–193.

WAKSMAN, S. A. (**1922**). Enzymes and microorganisms. *Abstracts Bact.* **6**:265–299, 331–360.

—— and DAVISON, W. C. (**1926**). *Enzymes.* pp. 57–58, 116. Williams & Wilkins, Baltimore.

WALDSCHMIDT-LEITZ, E., and MAYER, K. (**1935**). Über Amylo-phosphatase aus Gerste. *Z. physiol. Chem.* **236**:168–180.

—— and PURR, A. (**1931**). Über Amylokinase, einen natürlichen Aktivator des Stärkeabbaus in keimender Gerste. *Z. physiol. Chem.* **203**:117–131.

—— and —— (**1932**). Zur Kenntnis der Amylokinase. *Z. physiol. Chem.* **213**:63–70.

—— and REICHEL, M. (**1932a**). Zur Frage nach der chemischen Natur der Pankreas amylase. *Z. physiol. Chem.* **204**:197–203.

—— and —— (**1934**). Krystallisierte Hexaose aus Stärke. *Z. physiol. Chem.* **223**:76–80.

——, ——, and PURR, A. (**1932b**). Über α- und β-Amylase in Malz und Gerste. *Naturwissenschaften* **20**:254.

WALLERSTEIN, L. (**1939**). Enzyme preparations from microorganisms. Commercial production and industrial application. *Ind. Eng. Chem.* **31**:1218–1224.

WALTON, R. P. (**1928**). *A Comprehensive Survey of Starch Chemistry.* Vol. I, p. 230. Chem. Catalog Co., New York.

WEIDENHAGEN, R. (**1933**). Über die Reinigung pflanzlicher Amylasen. I. Mitteilung. *Z. Ver. deut. Zucker-ind.* **83**:505–515.

—— (**1936**). Beziehungen zwischen Vitamin C und enzymatischen Kohlenhydratspaltungen. 2 Mitteilung. *Z. Wirtschaftsgruppe Zucker-ind.* **86**:482–494.

—— and WOLF, A. (**1930**). Zur Kenntnis der Stärke. II. Über den enzymatischen Grenzabbau und das Komplement der Amylasen. *Z. Ver. deut. Zucker-ind.* **80**:866–876.

WERNER, G. D. (**1942**). A study of the purification and properties of the amylase of *Aspergillus oryzae*. *Dissertation,* Columbia University.

WIJSMAN, H. P. (**1889**). De Diastase beschouwd als mengsel. *Dissertation*, Amsterdam. (Through van Klinkenberg, 1932a.)

—— (**1890**). La diastase considerée comme un mélange de maltase et de dextrinase. *Rec. trav. chim.* **9**:1–13. (Through van Klinkenberg, 1932a.)

WILLSTÄTTER, R., WALDSCHMIDT-LEITZ, E., and HESSE, A. R. F. (**1923**) Über Pankreas-Amylase. *Z. physiol. Chem.* **126**:143–168.

WILSON, E. J., JR., SCHOCH, T. J., and HUDSON, C. S. (**1943**). The action of *macerans* amylase on the fractions from starch. *J. Am. Chem. Soc.* **65**:1380–1383.

WOHLGEMUTH, J. (**1908**). Über eine neue Methode zur quantitativen Bestimmung des diastatischen Ferments. *Biochem. Z.* **9**:1–9.

YAMAGISHI, G. (**1937**). Enzymic studies on cereals. VII. Dextrinizing amylase of rice seeds. *J. Agr. Chem. Soc. Japan* **13**:1268–1274.

CHAPTER III

APPLICATIONS OF THE AMYLASES IN MILLING AND BAKING TECHNOLOGY

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The industrial use of cereal amylases appears to be as old as civilization itself. Whether the first use coincided with the making of bread or with the production of alcoholic beverages is a debatable point. It has been stated that the earliest known records of the preparation of malt date back to 7000 B.C. and that beer brewing was an established craft by 5000 B.C. (Lazar, 1938). Certainly the art of breadmaking must be of equal antiquity.

The past century and a quarter has witnessed much research on the cereal amylases, and many reports have appeared dealing with the presence of these enzymes in cereals and their mode of action during breadmaking. In this chapter an attempt will be made to cite and review most of the pertinent literature on this subject. The discussion will include a brief historical treatment, followed by sections on the occurrence of amylases in baking materials, the degradation of starches by amylases, and the activities of these enzymes during baking. Amylase supplements are then considered, and the chapter ends with the discussion of modern concepts of the significance of determinations of amylase activity in flour and supplements. A number of reviews are available for supplementary reading: Rumsey (1922), Collatz (1922), Landis (1934), Hesse (1935), Landis and Frey (1936), and Blish, Sandstedt, and Kneen (1938).

THE HISTORIC BACKGROUND, 1816–1910

The proposal by Baker and Hulton (1908) that the "strength" of a flour was closely related to enzymatic properties had for its background a

number of discoveries. Kirchhoff (1816) found that boiled starch could be converted to sugar by the action of cereal grains. He further noted that this faculty was a property of the grain protein and that it was augmented by germination. Payen and Persoz (1833) named the active principle "diastase" and gave a considerable discussion of its properties. Märcker (1879) concluded from thermal and acid inactivation studies that there were two diastatic ferments in malt. Lintner (1887), in addition to confirming this concept, concluded that, whereas malt had two diastases, a starch liquefier and a starch saccharifier, ungerminated cereal had only the saccharifying type. This differentiation between the germinated and ungerminated cereals was expanded by Lintner and Eckhardt (1890) and by Brown and Morris (1890). Definite proof of the component concept was furnished by Wijsman (1890) and was further clarified by many subsequent investigators.

The application of the early knowledge of the diastase of cereals to breadmaking problems was quite clearly conceived by Jago (1886), who gave considerable data on the gassing power of doughs, the production of sugar during autolysis of flour–water mixtures, and the disappearance of sugar during fermentation. On page 155 of his book Jago writes: "The fermentation of dough is in part due to the fermentation of the sugar present, in part to the diastasis of a portion of the starch of the flour and its subsequent fermentation." At about the same time, Dünnenberger (1888) described in some detail the action of flour diastase in breadmaking and differentiated clearly between the chemical and microbial actions operative in the dough.

The foundation for our present concept of the role of alpha-amylase in breadmaking was laid by Jago (1895) in a second book. In a discussion of the value of malt supplements, he states (page 421): "The diastatic value of any preparation for breadmaking depends not simply on its activity as measured on soluble starch by Lintner's method, but on its power of converting starch paste, and even the imperfectly gelatinized starch occurring in bread." He adjusted diastases from malt and from wheat flour to the same Lintner value and used them as baking supplements. When compared with the unsupplemented control, bread baked with malt diastase showed a change in crumb characteristics and a greatly increased sugar content. In contrast, the use of supplemental flour diastase resulted in a loaf closely resembling the control. Neumann and Salecker (1908) likewise failed to find any relation between increases in loaf volume and the Lintner value of added malts, but did find a high correlation between volume response and increases in gas production obtained by adding malt to mixtures of either flour, yeast, and water or of wheat starch, yeast, and water.

An extensive treatment by Maurizio (1902) differentiated clearly between the gas-production and gas-retention factors that influence loaf volume. Humphries and co-workers (1907, 1909), Wood (1907), Ford and Guthrie (1908), Baker and Hulton (1908), von Liebig (1909), and Alway and Hartzell (1910) elaborated on this concept and established that at least two factors are operative in the production of a good loaf of bread: adequate diastatic activity to give enough sugar for ample gas production by the yeast (an important factor only when little or no sugar is added to the dough); and ability of the dough to retain this gas and produce a well-shaped loaf. Thus the principles underlying the relation of amylase activity and gluten quality to breadmaking were formulated; they require very little modification today.

THE AMYLASES IN WHEAT, FLOUR, AND GERMINATED WHEAT

In order to prepare for later discussion of the action of amylases in bread doughs, it is necessary to describe the amylases that are found in wheat flours and in malted wheat. As far as the flour is concerned, consideration will be limited to that made from sound, ungerminated wheat. Since rye and barley are similar in amylolytic properties to wheat, the discussion applies equally well to them (Chrzaszcz and Janicki, 1933; Kneen, 1944).

Occurrence of Amylases. Wheat and wheat malt are typified by the presence of two amylases. One of these, beta-amylase, is considered to be a saccharifying amylase. Its action on gelatinized starch results in the production of maltose by progressive release of terminal sugar molecules from the starch macromolecule. A residue of limit dextrin of high molecular weight remains, which stains purple with iodine and which imparts considerable viscosity to solutions. The other amylase, alpha-amylase, is regarded as a starch-liquefying and -dextrinizing enzyme. The viscosity of a starch paste is effectively reduced by its action, and dextrins of low molecular weight result from the splitting of internal linkages of the starch macromolecule. These dextrins are not colored by iodine, and their appearance is accompanied by only a very low production of fermentable sugars.

Sound ungerminated wheat has only very small amounts of alpha-amylase but relatively large amounts of beta-amylase. A considerable proportion of both these enzymes is in a form not readily extracted by water (latent or bound amylase). This latent amylase is released by the action of proteolytic enzymes (*e.g.*, papain) or to some extent by the peptizing action of salts. The aggregate of the amylase readily extracted

with water or very dilute salt solution (free amylase) and the latent amylase is usually referred to as total amylase. When wheat is germinated, a progressive release of the latent beta-amylase occurs coincidently with a rise in alpha-amylase activity to the high levels typical of wheat malt. These characteristics of wheat and germinated wheat are illustrated by Table I, containing data from Kneen, Miller, and Sandstedt (1942).

TABLE I

THE AMYLASES OF UNGERMINATED AND GERMINATED WHEAT (Adapted from Kneen, Miller, and Sandstedt, 1942)

Days germinated[1]	Beta-amylase activity		Alpha-amylase activity	
	"Free"[2]	"Total"[3]	"Free"[2]	"Total"[3]
	units	*units*	*units*	*units*
0	10.1	28.4	0.027	0.043
3	16.3	28.6	35.0	40.5
4	20.8	26.5	87.2	90.7
5	26.6	26.2	229.0	238.0

[1] Germination temperature, 15 °C.
[2] "Free" extract, one hour with dilute calcium acetate.
[3] "Total" extract, eighteen hours with papain.

In the sample of wheat used to obtain the data of Table I, roughly one-third of the beta-amylase and one-half the alpha-amylase was free. It should be noted that, whereas the free beta-amylase increased by germination to a level equal to the total papain-extractable beta-amylase, the total itself did not increase but showed a slight decrease. In contrast, both the free and total alpha-amylase showed progressive increases in activity throughout the germination period. From these and other considerations it appears that beta-amylase is merely activated or released by germination, but that an actual production or synthesis of alpha-amylase occurs.

Wheat amylases have not received the extensive study that has been devoted to those of barley, but there is sufficient evidence to indicate that the amylases of wheat depend on both variety and environment of growth. Elizarova (1940) found that spring wheats had more beta-amylase than winter wheats. Data relating to the hard red winter wheats indicate that beta-amylase content is influenced by variety and environment of growth (Kneen and Hads, 1945). Within a variety the protein content was found to be correlated with the amount of total papain-extractable beta-amylase but not with the fraction readily soluble in water.

As the amylase system of a wheat flour reflects that of the grain from which it was milled, it would be anticipated that the beta-amylase content of flours would also show considerable variation. Dadswell and Wragge

(1940) found wide variations in the water-soluble beta-amylase of a number of Australian bread flours. In an extensive study of flours from Canadian hard red spring wheats, Meredith, Eva, and Anderson (1944) found that the varietal means for beta-amylase activity (total saccharifying activity of papain extracts) varied from 42.1 to 26.8 units.

Brief mention may be made of other enzymes with amylase-like properties that have been postulated as operating in the starch-degrading actions of flour and malt. In 1890, Brown and Morris conceived that a cytolytic enzyme functioned in breaking down the outer covering of native starch granules, which was supposed to be very resistant. This action was postulated as preliminary to amylase attack. A somewhat different type of action was proposed by Blish, Sandstedt, and Mecham (1937) with the hypothesis of a "raw starch amylase" with distinctive properties. The recent application of more refined techniques to malt analysis (Kneen, Beckord, and Sandstedt, 1941) has indicated that the action of malt on raw (native) starch parallels the starch-degrading actions commonly attributed to alpha-amylase, which suggests that the existence of a separate "raw starch amylase" is questionable.

Another starch-degrading enzyme, amylophosphatase, with properties similar to alpha-amylase has been described. The data relating to this enzyme have been summarized by Mayer (1939). It was postulated that amylophosphatase liquefies starch pastes with inappreciable production of dextrins and reducing groups but with the liberation of phosphates. Neither the practical significance of this enzyme nor the differentiation of its action from that of alpha-amylase has been clearly defined.

Starch degradation can be explained by assuming that only two amylases are operative, alpha-amylase and beta-amylase. However, the system may not be this simple. Other amylases may exist with properties different from those of these two components. In addition, the conversion of starch to sugars may be influenced by enzymes with properties distinct from those of the amylases but effective in the degradation of sugar polymers other than starch. Examples of the possible complexity of the system are evidenced by the discovery in plants and animals of a phosphorylase that catalyzes the reversible system, starch (or glycogen) $\rightleftharpoons$ glucose-1-phosphate (Cori and Cori, 1940; Hanes, 1940, 1940a), and the report by Kneen (1945) that sorghum malt contains a "glycosidase" that supplements the actions of alpha- and beta-amylase in starch conversion. While the discussion of the degradation of starch in dough will be concerned mainly with the actions of alpha- and beta-amylase, it should be kept in mind that the processes may be much more complex than indicated.

Measurement of Amylase Activity. The definition of alpha-amylase as a starch-liquefying and -dextrinizing enzyme and of beta-amylase as a

saccharifying enzyme is an indication of the methods used for the measurement of their activities. However, in the interpretation of the results obtained by such measurements, the interrelationships between the actions of the two components must be recognized (Kneen, Beckord, and Sandstedt, 1941). In addition to those methods that measure the over-all actions of the mixtures, methods are available for the estimation of the individual amylase components. In all instances, the materials to be evaluated are extracted with a suitable solvent and the activities on standard gelatinized starch substrates are determined.

The beta-amylase content of a wheat flour can be evaluated by the well-known method of Lintner (1886) or by modifications of this method such as those proposed by Anderson and Sallans (1937) or by Kneen and Sandstedt (1941). A suitable aliquot of the extract is allowed to react with boiled soluble starch under standard conditions. The production of reducing groups is evaluated and the results expressed in appropriate units. The alpha-amylase activity of flour extracts is usually too low to influence this reaction and the values obtained for saccharification represent beta-amylase activity. With germinated wheats the same method is used, but the saccharifying activity reflects the combined actions of the two amylases. A correction for alpha-amylase activity may be applied in the manner proposed by Kneen and Sandstedt (1941), and saccharification due solely to the beta component may thus be calculated.

Two types of techniques are available for the evaluation of alpha-amylase activity, one based on the ability of this enzyme to liquefy viscous starch pastes, the other based on dextrinizing power. A liquefaction procedure such as that proposed by Józsa and Johnston (1935) or by Blom and Bak (1938) is applicable in the measurement of the small amounts of alpha-amylase present in flour extracts. The rate of reduction in the viscosity of the paste is determined under standard conditions and the results expressed in appropriate units. Apparently the values are not influenced by the presence of beta-amylase. A micro dextrinization procedure was proposed by Kneen, Sandstedt, and Hollenbeck (1943) for the evaluation of flour alpha-amylase. This technique involves a prolonged digestion of soluble starch in the presence of an excess of beta-amylase, followed by rapid dextrinization with a larger amount of alpha-amylase of known activity. From the results, the dextrinizing activity of the minute quantity of alpha-amylase in a flour extract may be calculated. With germinated wheat, either the liquefaction or the dextrinization technique is applicable. For the determination of dextrinizing activity, the macro method of Sandstedt, Kneen, and Blish (1939) was devised. By carrying out the reaction in the presence of an excess of beta-amylase, the results reflect only the variation in dextrinization rate attributable to variation in alpha-amylase

activity. The alpha-amylase unit values that may thus be calculated are specific for this component as compared with the dextrinizing units obtained in the absence of an excess of beta-amylase; these latter, like saccharifying (Lintner) units, evaluate the combined actions of both amylase components.

Autolytic Evaluations. Distinct from the methods designed to measure directly the potential starch-degrading power of the amylases in flour and malt are those techniques involving a simple digestion of the flour with water, either as a suspension or as a dough. Perhaps the first techniques set up specifically to evaluate the amylase (diastatic) activity of flours were those described in the new chemical test for "strength in wheat flour" proposed by Wood (1907a). He suggested two methods that, in essentials, vary little from those used today. One of the methods involved the autolysis of flour in water under conditions (3 hours at 30°C) similar to those used in baking. Sugar content was determined at the end of a specified period, and this value less the blank was considered a measure of amylase activity. The companion technique was similar in principle but modified by the incorporation of yeast with the flour and water and by the indirect evaluation of autolytic sugar production from the carbon dioxide evolved. Here too a blank representing the sugar initially present in the flour was subtracted in order to arrive at the true amylase activity. The stimulation of autolytic sugar production resulting from malt supplements was observed and the importance of diastase action appreciated.

Six years later, Swanson and Calvin (1913) made an extensive investigation of the influence of temperature, time, added chemicals, and the ratio of flour to water on the production of sugar in flour–water mixtures. But it remained for Rumsey (1922) to popularize the autolytic method for evaluating the diastatic power of flours. Rumsey standardized the conditions of measurement and proposed that the quantity of maltose produced be designated as the unit for evaluation of amylase activity; this value is called the "maltose figure" throughout this chapter. With the exception of the introduction of a buffer system (Malloch, 1929), improvements of the method have been based on facilitating the chemical measurement of the maltose produced. This sugar can be measured by polarimetric or refractometric techniques, or by the reducing action on such oxidizing agents as alkaline solutions of copper sulfate or potassium ferricyanide. A technique that has gained wide popularity is based on the reduction of ferricyanide; it was proposed in its present form by Blish and Sandstedt (1933).

The other technique proposed by Wood (1907a) for evaluating amylase activity in flour was standardized and adapted to laboratory procedure. Here too it was necessary to specify definite conditions for the flour–water–yeast ratios and for the time and temperature of the reaction. The gas production may be measured volumetrically, manometrically, or by ab-

sorption (chemical reaction). As examples of the first two types of measurement, the volumetric procedure of Bailey and Johnson (1924) and the manometric procedure of Sandstedt and Blish (1934) may be cited.

The fundamental relation between gas production and maltose figure has been studied frequently (Blish, Sandstedt, and Astleford, 1932; Sandstedt, 1934; Davis and Worley, 1934; Berliner, 1936; Eva, Geddes, and Frisell, 1937; Bottomley, 1938). The maltose figure is arrived at by a one-hour autolysis and provides no record of the fermentable nonreducing sugar present in the flour. The gas-production method more nearly simulates the conditions of the baking procedures by extending the period of autolysis to several hours and by recording all the fermentable sugars originally present as well as those produced. By and large the values given by the two methods are highly correlated. Nevertheless, gas production cannot be predicted accurately from the maltose figure unless the period of autolysis is extended to a comparable time and the initial content of nonreducing sugar is included in the evaluation (Sandstedt, 1934).

The rate of sugar production during the autolysis of a flour–water mixture does not provide an evaluation of amylase content; rather, it reflects an activity under limiting conditions. Flour contains a variable percentage of damaged starch granules. Beta-amylase can attack only the damaged granules; and, as there is ample enzyme by comparison with the small fraction of susceptible substrate, activity is limited not by enzyme content but by the quantity of damaged granules. Following the observation of Alsberg and Griffing (1925) that fine grinding of flour increased starch conversion, this concept of the dependence of autolytic measurements (maltose figures) on both the substrate and the enzyme was elaborated by such workers as Mangels (1926), Malloch (1929a), Karacsonyi and Bailey (1930), Pascoe, Gortner, and Sherwood (1930), Johnson (1930), Landis and Frey (1933), and Sandstedt, Blish, Mecham, and Bode (1937). The variety of the wheat milled and the environment in which the grain was grown were shown to be important factors by Mangels (1926), Markley and Bailey (1934), Swanson (1935), Leatherock, McGhee, and Giertz (1937), Schleip (1938), Knyaginichev, Mutul, and Palilova (1940), and Dadswell and Wragge (1940). Data relating to the injury of starch during milling and the influence of this injury on autolytic maltose production were presented adequately by Leatherock and co-workers (1937), Ziegler (1940), Jones (1940), and Dadswell and Wragge (1940). The last-named workers demonstrated that maltose production was correlated not with enzyme content but with the starch injury resulting from milling. This confirmed the conclusion of Jones (1940), who found the content of a certain type of damaged starch granules, "ghosts," to be closely related to the maltose figure.

The transition is now complete; it has progressed from the earliest concept, that one-hour autolytic values measured mainly enzyme content, through a phase in which the influence of the starch substrate was regarded as more and more significant, to the present concept that such values reflect mainly the susceptibility of flour starch.

Consideration of the influence of amylase activity on the maltose figures of sound flours has been limited to the role of beta-amylase. Though alpha-amylase occurs in these flours in relatively minute quantities, variations in the activity of this enzyme may cause measurable differences in autolytic sugar production; but far greater differences are caused by variations in the amount of starch damage. It becomes apparent that the terms "diastatic activity" and "diastatic power" are misnomers, since the activity of "diastase" may be a minor factor influencing the evaluation. The terms "maltose figure," "maltose value" or "one-hour saccharogenic value" are more descriptive.

DEGRADATION OF STARCHES BY AMYLASES

The products resulting from amylase action on starch, and the quantities produced in a given period of time, are dependent on the nature of the substrate and on the nature of the enzyme. The actions on two types of substrate will be considered: the degradation of native (undamaged) starch granules, and the degradation of gelatinized (available) starch.* Three types of action on this latter substrate are considered: liquefaction, dextrinization, and saccharification. With respect to the action of cereal enzymes on starch, all these types of degradation may be considered as brought about, primarily at least, by two amylases, alpha and beta, acting alone or in combination.

Degradation of Native Starch. Commercially milled wheat flours vary greatly in the amount of damaged starch granules. In dividing the wheats into classes, it can be roughly estimated from maltose figures that from 1 to 2 per cent of a soft wheat flour consists of this fraction, from 3 to 4 per cent of a hard wheat flour, and from 6 to 8 per cent of a durum flour. It is apparent that the greater portion of the starch in any flour is composed of uninjured granules. Though the resistance of such granules to degradation by amylases was noted by early investigators (*e.g.*, Brown and Heron, 1879; Brown and Morris, 1890; Stone, 1896), it is only recently that

* The large percentage of the granules of flour starch are undamaged and may be designated as native starch granules. Starch damage ranges through various degrees from slight to severe. The starch of the more severely damaged, or the disintegrated, granules gelatinizes in water and in such form is relatively susceptible to amylase action.

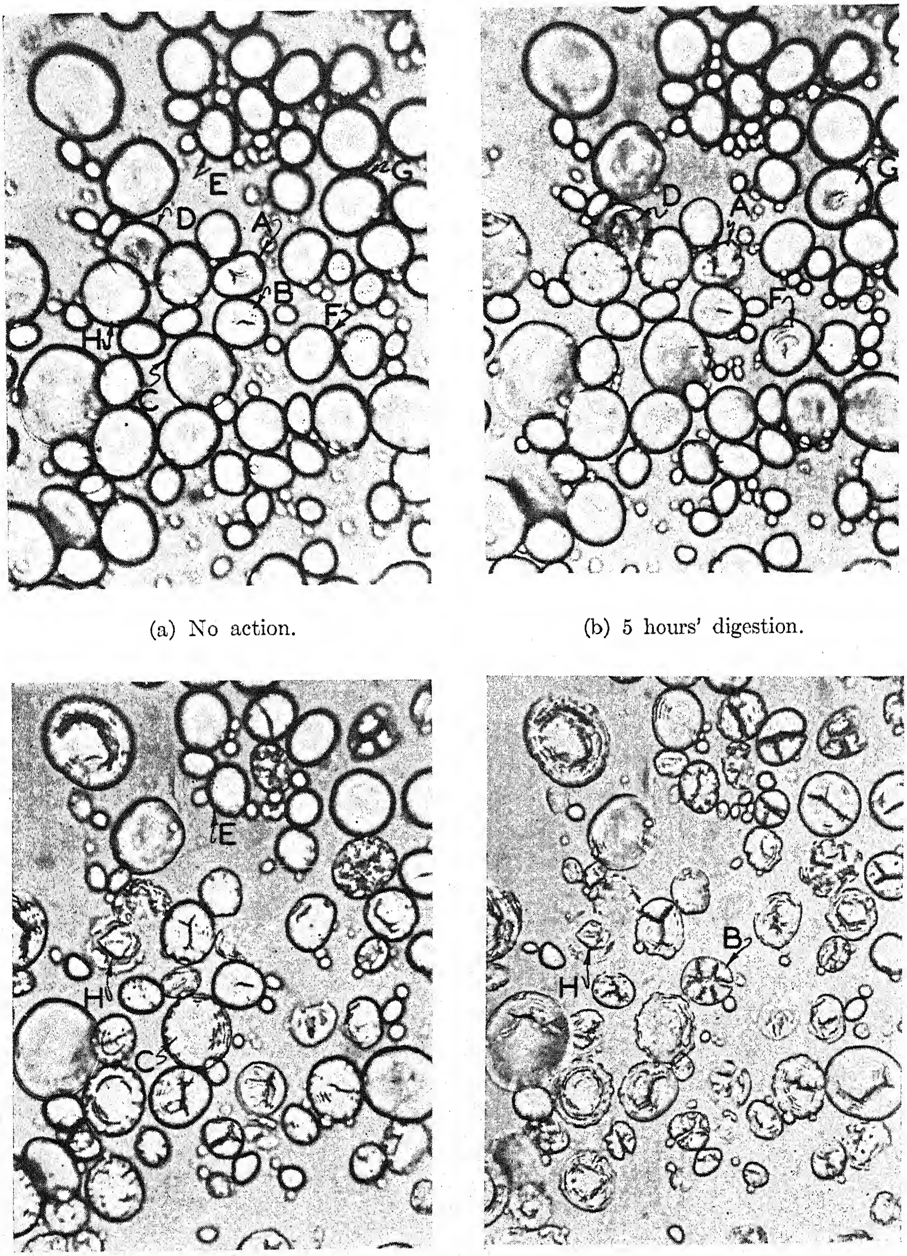
much attention has been devoted to studies of this action and to its possible significance in breadmaking.

Sandstedt, Blish, Mecham, and Bode (1937) showed that the undamaged starch granules of flour are not attacked by beta-amylase, whereas the damaged fraction is readily available to this enzyme. The data of Stamberg and Bailey (1939) provide further confirmation of the resistance of native starch to beta-amylase action. On the other hand, it was shown by Blish, Sandstedt, and Mecham (1937) that wheat flour does contain an enzyme capable of degrading undamaged starch granules and these workers postulated the enzyme to be a "raw starch amylase" not identical with either alpha- or beta-amylase. Its properties were found to be similar to those of alpha-amylase, and as with alpha-amylase there was a marked increase in activity when the wheat was malted.

Native starches vary greatly in their resistance to the action of malt amylases. For example, in the microscopic examination of granules of potato starch that have been digested for several days with malt extract, practically no signs of starch degradation are evident. On the other hand, wheat starch granules show considerable degradation when digested under similar conditions. The difference in resistance between native and either gelatinized or damaged starch is even more marked; the native granules are, by comparison, exceedingly resistant.

The manner and rate of the action of wheat malt amylases on wheat starch granules are illustrated in Figure 1, taken from Sandstedt (1941). Figure 1a shows the appearance of a sample of starch immediately after the addition of a 1:5 extract of wheat malt in water, buffered at *p*H 4.7, and containing calcium acetate for stabilization of alpha-amylase. Certain granules are designated by letters and, the field being the same in each case, may be followed throughout the series. Figure 1b shows the results of 5 hours' digestion at room temperature. Granules *A*, *D*, *F*, and *G* apparently were damaged in various ways and already showed considerable disintegration. The most rapid action on these granules was confined to the region of injury and the rate of such action was determined by the severity of the damage. At this 5-hour stage no action was apparent on most of the undamaged granules.

Amylase action had progressed considerably after 120 hours (Fig. 1c) and degradation was evident even with most of the more resistant granules. This degradation of resistant granules is typical of the action of malt alpha-amylase on undamaged wheat starch granules. The action begins at the periphery of the granule and progresses toward the center, spreading tangentially at each lamina at a rate dependent on the susceptibility of the particular lamina (granule *C*, Fig. 1c). In most cases the periphery is the weak point in the granule but other surfaces also are sub-



(a) No action.

(b) 5 hours' digestion.

(c) 120 hours' digestion.

(d) 264 hours' digestion.

Fig. 1.—The action of wheat malt amylases on wheat starch granules.

ject to attack; in some cases these other surfaces are acted on before peripheral action commences (granule *E*, Fig. 1c).

The great variability in susceptibility of individual starch granules is well illustrated in Figures 1c and d. Many granules showed no evidence of degradation after 120 hours of digestion; some small granules showed none even after 264 hours. Frequently portions of granules (*e.g.*, portions of granule *H*) exhibited more resistance to amylase action than did other whole granules. The fact that granule *B* showed a high degree of resistance, notwithstanding apparent extensive damage, further illustrates the complexity of the degradation of starch granules by amylases.

The degradation of native starch by amylases is a relatively slow process and one influenced but little by beta-amylase. When starch is gelatinized, for example, by heat, or mechanically injured, it becomes available to beta-amylase and at the same time is more susceptible to attack by alpha-amylase. The fundamentals of the well-known actions of alpha- and beta-amylase on boiled starch apply in the conversion of the "available" fraction of flour starch during fermentation and in the degradation of starch gelatinized by oven temperatures.

Degradation of Gelatinized Starch. Starch may be treated in various ways in the preparation of a substrate for enzyme action, *e.g.*, by dry grinding, by heating in water to the gelatinization point, or by alkali gelatinization. A frequently used type is known as "soluble starch" or "soluble starch according to Lintner." When a water suspension of this modified starch is boiled, a substrate of low viscosity and well adapted to studies of dextrinization and saccharification is obtained. On the other hand, the viscous paste formed by the gelatinization of potato starch is the customary substrate for investigations of the liquefying activity of amylase. The manner of preparing the gelatinized starch substrate may vary with the amylase action to be studied, but the products have in common a greatly enhanced susceptibility to amylase action over that shown by the native granules.

The viscosity of a starch paste is rapidly reduced by the action of relatively small amounts of alpha-amylase. This starch *liquefaction* is primarily a function of the alpha component (Józsa and Johnston, 1935; Kneen, Beckord, and Sandstedt, 1941; Hollenbeck and Blish, 1941). In the early stages, disaggregation is evident, and inappreciable production of dextrins or reducing groups occurs. During the later phases of liquefaction, disaggregation merges into the typical splitting of starch molecules to dextrins and the liberation of reducing groups, *i.e.*, dextrinization and saccharification.

Either alpha- or beta-amylase may cause the *dextrinization* of gelatinized starch. Both are capable of splitting starch to dextrins and sugar

but they differ markedly in the type of end-products produced. Alpha-amylase converts starch to small dextrins which give no color reactions with iodine, and this is coincident with a minor production of fermentable sugars. In contrast, the action of beta-amylase is typified by the production of end-products composed of a complex "limit dextrin" (frequently designated as alpha-amylodextrin), and a fermentable sugar. With common blue staining starches, limit conversion by beta-amylase involves maltose production equivalent to about 60 per cent of the starch. Since both alpha- and beta-amylase have dextrinogenic activities, it would be expected that the reduction of starch to these less complex fragments would be achieved more rapidly when both enzymes are active than in the presence of either one alone. This has been demonstrated by Blom, Bak, and Braae (1937), Hanes and Cattle (1938), and Sandstedt, Kneen, and Blish (1939).

Reducing sugars usually result from the action of amylases on starch. The *saccharification* is considered as typical of conversion achieved by either beta-amylase alone or by combinations of alpha- and beta-amylase. However, the independent action of alpha-amylase results in the production of fermentable sugar, and fairly high levels of starch saccharification apparently may be achieved even in the absence of beta-amylase. As in dextrinization, beta-amylase is inefficient in saccharification, since the sugar resulting from its action represents only about 60 per cent of the starch present. The rapid and relatively complete conversion of starch to fermentable sugar, typical of the action of barley or wheat malt, is a consequence of the combined actions of the alpha- and beta-amylase they contain.

The interrelationships of the amylases in various types of starch degradation have been outlined by Kneen, Beckord, and Sandstedt (1941) and need not be further discussed here. From a practical standpoint the important concepts are that liquefaction, dextrinization, and saccharification of gelatinized starch, and probably the degradation of native starch, all are dependent on the presence of alpha-amylase for maximum efficiency. The rates of dextrinization and saccharification, and probably the conversion levels, are greatly increased by the supplementary presence of beta-amylase. This beta component, when unaccompanied by alpha-amylase, is inefficient in the liquefaction, dextrinization, and saccharification of gelatinized starch and apparently is incapable of degrading native starch.

AMYLASE ACTIVITY DURING BREADMAKING

The manner in which amylases are operative in bread doughs can best be considered by dividing the breadmaking process into two phases,

fermentation and baking. The conversion of starch to sugars during dough fermentation has been the object of much research but little has been devoted to studies of the action of amylases during baking. Accordingly, though the one series of reactions can be considered with some assurance, a discussion of those occurring in the later phase must be somewhat hypothetical. Nevertheless, enough is known of the properties of the amylases and of wheat starch to formulate a concept of the type of degradation which may take place as dough temperatures pass through the gelatinization range of the starch and the inactivation ranges of the amylases. Bread doughs that have received no diastatic supplement are deficient in, but usually not free from, alpha-amylase, whereas supplemented doughs contain additional alpha- and beta-amylase. The discussion will concern chiefly doughs that have been diastatically supplemented. The same actions are operative in unsupplemented doughs but, as far as alpha-amylase action is concerned, to a lesser degree.

Before discussing the specific behavior of amylases in breadmaking, it is necessary to outline a few general principles that govern amylase action in doughs. As with enzymes in general, the amylases are influenced by the hydrogen-ion concentration of the medium, by temperature, and by other factors such as activators and inhibitors. Activity and stability factors should be clearly differentiated since starch degradation is influenced not only by the activity of the enzyme during a short interval of time but by the maintenance of that activity over longer periods and under conditions frequently conducive to an irreversible loss of enzyme. For example, the optimum hydrogen-ion concentration for alpha-amylase activity at room temperature and for short periods of time is in the neighborhood of pH 5.0, whereas the optimum for stability is close to pH 7.0. On the other hand, beta-amylase has a stability optimum more nearly approaching its activity optimum.

It is difficult to assign exact figures representing optimal hydrogen-ion concentrations for the actions of the amylases. The concentration at which maximum activity will be apparent is influenced by the temperature used and by the time factor involved. Under usual doughing conditions, in the neighborhood of 30°C for several hours, the optimum for the amylases of dough lies in the range pH 4.5 to 5.5. Similarly there is no definite optimum temperature for activity. An increase in temperature from, say, 20 to 30°C approximately doubles the activity of the amylases. As the temperature is further raised, the increase in activity becomes progressively less until a point is reached at which no increase is achieved by a slight rise in temperature. This point has been described as "a temperature optimum," but it represents nothing more than a balance between the rate of increase in enzyme activity and the rate of irreversible loss of the enzyme.

As the temperature is raised still further, enzyme activity starts to drop off and, eventually, depending on the time and temperature, all activity is lost. At a dough temperature of 30°C, the action of the amylases is quite rapid and loss of activity is slow. When the dough goes to the oven there is an increase in activity, which soon levels off and then decreases rapidly as internal loaf temperatures approach 80°C.

Certain substances such as salts and protein degradation products have been described as amylase activators (*cf.* Sherman and co-workers, 1915, 1921, 1928, 1930). Such substances may occur in doughs and may increase the apparent activity of the amylases. In some instances, as in the effect of calcium ions on alpha-amylase (Kneen, Sandstedt, and Hollenbeck, 1943), the action is a stabilizing one; in others, it is probable that the substances are effective in increasing the "solubility" or availability of the amylases. The significance of these factors in baking is not well understood but it is conceivable that they may have a pronounced influence.

Some amylase inhibitors have also been described. The end-products of amylase action, maltose for example, are regarded as inhibitory to further action of the enzyme. The removal of these by yeast fermentation in doughs should therefore tend to maintain high rates of action. The role in breadmaking of specific inhibitory substances, such as the insoluble substance described by Chrzaszcz and Janicki (1933a, 1934), or the water-soluble, protein-like inhibitor described by Kneen and Sandstedt (1943), has not been determined, but it is doubtful whether much significance can be attributed to their action. For example, the inhibitor of Kneen and Sandstedt (1943) appears to be specific for animal and certain bacterial amylases but has no effect on cereal amylases.

Starch Degradation in the Fermenting Dough. For the present purpose, the period of dough fermentation is considered to encompass the entire period between the beginning of mixing and the end of the proof period. As the dough ingredients are added together enzymatic action commences. The small fraction of the wheat starch that has been severely injured by milling is characterized by a high degree of availability for degradation by amylases and by a high water-holding capacity. Liquefaction of this fraction by alpha-amylase, in addition to influencing its capacity for water retention, renders it more susceptible to attack by beta-amylase. Dextrinization and saccharification follow and the net observable result, especially in flours containing large quantities of damaged starch, is a decrease in water absorption capacity, a slackening of the dough, and the development of stickiness. These are emphasized by heavy diastatic supplementation. In those unsupplemented doughs having a natural deficiency of alpha-amylase, the saccharification is limited to that caused by beta-amylase, and little loss of water-holding capacity occurs; but, with the

levels of alpha-amylase achieved by adding supplements, conversion of available starch proceeds to a much greater degree. In addition to the available fraction, some of the starch may be considered as borderline material. Alpha-amylase degrades it at a rate dependent on the severity of the damage, but it is resistant to the action of beta-amylase unaccompanied by alpha-amylase. Under the influence of the excess of beta-amylase present in dough and the small amounts of alpha-amylase contributed by a diastatic supplement, a rapid saccharification occurs, the major part of the conversion being achieved during the early stages of fermentation.

The production of sugars by amylases during the first part of fermentation is supplemented by degradation of the slightly injured and the uninjured starch granules. This action is entirely dependent either on alpha-amylase action or on the action of an enzyme closely associated with alpha-amylase. The relative amounts of sugar produced by this type of degradation during the first stage of fermentation are insignificant compared with the rapid sugar production from damaged starch. However, once the available fraction is used up, any sugar production occurring must be attributed to conversion of undamaged starch. There are ample data (Sandstedt, Blish, Mecham, and Bode, 1937; Blish, Sandstedt, and Mecham, 1937) to show that the rate of sugar production from undamaged starch under any normal circumstances is too slow to have much influence on the sugar levels of the dough.

Some of the physical changes in dough properties that accompany the early phases of diastasis and fermentation are usually attributed to the removal of starch. In the absence of conclusive evidence it is reasonable to assume that at least part of the difference in dough-handling properties between unsupplemented and supplemented doughs may depend on the type of dextrin present. The persistence of beta-amylase limit dextrin (alpha-amylodextrin) to the extent of some 40 per cent of the available starch fraction is a characteristic of doughs made from flours deficient in alpha-amylase. This dextrin may not occur at all, or at least is greatly reduced in quantity when alpha-amylase is operative.

Starch Degradation during Baking. Enzyme action during the fermentation period accomplishes a depletion of the available starch to a point at which further sugar production is practically restricted to the degradation of relatively sound granules. The rate of action on these granules increases rapidly as the dough temperature approaches the gelatinization region, but the starch conversion produced by this action must still be of minor significance compared with that resulting in the gelatinization range. Primarily, the degree of degradation achieved by amylase action in the oven will depend on the rate at which the dough temperature passes through the gelatinization range for wheat starch, the hydrogen-ion concentration of

the dough, the presence or absence of amylase-stabilizing factors, and the quantity and kind of amylases present.

It would be expected that a bread dough produced by either straight dough or sponge procedure would have approximately the same effective amylase content at the end of the normal fermentation period as at mixing. The hydrogen-ion concentration of the dough increases during fermentation, but even in a sponge it should not reach a point much below *p*H 5.0 (Bailey and Sherwood, 1923) and at the time of baking may approximate *p*H 5.5. The actual concentration will depend on the buffer capacity of the flour, the type of supplement used, and the fermentation time. The hydrogen-ion concentrations cited are above the range considered optimum for alpha-amylase stability but are conducive to the retention of beta-amylase activity (Kneen, Sandstedt, and Hollenbeck, 1943). Retention of alpha-amylase activity under these conditions (*p*H 5.0 and 30°C) will be influenced primarily by the calcium-ion content of the dough. The amounts of calcium salts present in flours, together with those added in tap water and in some types of "improvers," insure fairly adequate levels in commercial practice.

In many laboratory procedures, distilled water is used without adding calcium ions. A marked loss of alpha-amylase during doughing and baking may well occur under these conditions. The advisability of routine addition of calcium salts in experimental baking would bear some investigation. Adding calcium salts would certainly tend to insure the alpha-amylase stability usually operative in commercial doughs. Though the calcium ion is an instability factor for beta-amylase (Kneen, Sandstedt, and Hollenbeck, 1943), little if any loss of this component would be expected in practice.

As the bread dough goes to the oven it is characterized by an alpha-amylase activity dependent primarily on the amount of this component previously in the flour plus that added by supplementation. An abundance of beta-amylase is present; in fact, the amount of active beta-amylase potentially operative at this stage should show an increase over the original owing to the release of part of the "bound" fraction during fermentation. The dough therefore has the potentialities for starch degradation by amylase in the oven, but the amount of degradation will be influenced by modifying factors, particularly temperature, operating during baking.

Studies have been made of internal dough temperatures during baking (*e.g.*, Bailey and Munz, 1938), and of the hydrogen-ion concentration in dough and bread (Bailey and Sherwood, 1923; Grewe and Bailey, 1927; Fisher and Halton, 1929; Blish and Hughes, 1932). The indications are that bread dough goes to the oven at between *p*H 5.0 and *p*H 5.5, that the level is likely to be close to the higher figure, and that a slight increase oc-

curs during baking. The data of Bailey and Munz (1938) show that from 3 to 4 minutes are required for the internal temperature of 500-g loaves to pass through the range from 60 to 75°C.

The findings of Kneen, Sandstedt, and Hollenbeck (1943) indicate that, at a hydrogen-ion concentration between *p*H 5.0 and 5.5, alpha-amylase activity will be lost quite rapidly, especially at high temperatures. However, in the presence of an adequate calcium-ion concentration, sufficient activity should remain to be effective in the temperature range for gelatinization of wheat starch (60 to 75°C). This hydrogen-ion concentration is satisfactory for the retention of beta-amylase activity; the presence of calcium ions will speed up the loss of this component at high temperatures but the initial large excess again insures some action.

The type of reaction between wheat starch and the amylases in the temperature range of 60 to 75°C may be postulated from fundamental considerations. Between 60° and 65° the susceptibility of the starch increases markedly, and in the neighborhood of 65° gelatinization is evident. The gelatinizing starch is rapidly liquefied by alpha-amylase, then converted in part to dextrins by the combined action of the two amylase components. Simultaneously, a production of maltose may occur, again brought about by the action of the two components. The rapid increase in dough temperature up to and past 70° brings about a decrease in activity and finally complete inactivation of beta-amylase. The action of alpha-amylase persists at the higher temperature levels, 70 to 75°, but decreases greatly in effectiveness, to be completely lost in the neighborhood of 75°.

It is apparent that a considerable proportion of the starch might be liquefied in the presence of an adequate alpha-amylase supplement. With normal supplementation the degree of dextrinization should be marked. Production of maltose from the gelatinized starch and from the dextrins may occur but in lesser degree. If the dough has excessive alpha-amylase activity—*e.g.*, if it is oversupplemented or made from sprouted wheat—conversion of starch to dextrins and maltose will be much more pronounced and may approach levels decidedly detrimental to the quality of the baked loaf (Kozmin, 1933; Freeman and Ford, 1941).

Kozmin (1933) has provided ample evidence that, in the baking of dough made from the flour of sprouted wheat, a great increase of dextrins occurs over that contained in the fermented dough. It was found that the increase was of much greater magnitude than the normal increase occurring in the baking of doughs from sound wheat flours. This is illustrated by Table II from Kozmin's paper. Two flours were used, one (No. 5) milled from an ungerminated wheat, the other (No. 6) from the same wheat after germination. The maltose figures (Rumsey method) for the two flours were 286 and 652 units, respectively. It is apparent that the ungerminated

wheat was such that a great deal of starch damage occurred during milling. It would be expected that the development of alpha-amylase by germination would cause an increase in the maltose figure. As pointed out by Kozmin (1933), baking, even of the unsupplemented flour, effected a pronounced increase in the content of water-soluble intermediate products of starch hydrolysis. This starch hydrolysis in the oven was greatly augmented with the heavily supplemented flour. It is significant that the excessive amylase activity during the fermentation of the flour made from germinated wheat led to a considerable residue of maltose in the dough, whereas the transformation from dough to bread was not effective in the production of maltose. Lack of maltose production in the oven supports the previous postulate that the effective amylase during baking is the relatively thermostable liquefying and dextrinizing component, alpha-amylase.

TABLE II

CHEMICAL COMPOSITION OF THE WATER-SOLUBLE FRACTION OF THE DOUGH IMMEDIATELY BEFORE GOING TO THE OVEN AND OF THE BREAD, AS PER CENT OF DRY WEIGHT (From Kozmin, 1933)

Flour No.	Source	Composition	Experiment 1		Experiment 2	
			Dough	Bread	Dough	Bread
5	Ungerminated wheat	Water-soluble substances	9.81	16.80	7.70	12.74
		Protein (N × 6.25)	1.94	1.00	1.84	1.26
		Maltose	—	2.40	2.14	2.81
		Other non-nitrogenous substances	7.87	13.40	3.72	8.67
6	Same wheat after germination	Water-soluble substances	21.26	46.00	18.00	43.00
		Protein (N × 6.25)	2.25	2.00	2.27	1.23
		Maltose	6.90	9.40	7.00	5.63
		Other non-nitrogenous substances	12.11	34.60	8.73	36.14

Kent-Jones and Amos (1940) discussed Kozmin's data and proposed that a "dextrin figure" be determined for flours in order to assess the alpha-amylase activity. These authors pointed out that dextrinization becomes a problem of great significance in doughs that are baked slowly. As has been suggested above, the rate of destruction of alpha-amylase at high temperatures is dependent on the hydrogen-ion concentration of the dough and on the rate at which the destructive temperatures are approached and passed through. If the rate is slow, much starch hydrolysis may occur before complete inactivation of the amylases occurs. Kozmin (1933) reported that the excessive starch hydrolysis in overdiastated doughs could be prevented by acidification to *p*H 4.4. The data of Kneen, Sandstedt, and Hollenbeck (1943) indicate that *p*H 4.4 should be effective in the irreversible inactivation of alpha-amylase not only in the range of oven temperatures but also

at fermentation temperatures. It seems certain that the rate of loss of alpha-amylase under these conditions is markedly influenced by the amount of stabilizing agents, such as calcium ions, present in the dough.

Further confirmation of the concept that amylase action is effective in the oven is provided by the data of Freeman and Ford (1941), who found that bread baked from supplemented dough showed a pronounced increase in dextrins and maltose over an unsupplemented control. The production of maltose during baking was marked but of no greater degree in the malt-supplemented than in the unsupplemented doughs; this again indicates that the changes in loaf quality resulting from malt supplements are a function of alpha-amylase.

The foregoing discussion has been concerned with amylase action in breadmaking. In the production of other types of baked goods from wheat flour many modifications are involved, and some of these may influence amylase activity and the destruction of amylases during processing. No attempt will be made to deal with these in detail. From a knowledge of the fundamentals of amylase action on starch, and a knowledge of the influence of such factors as temperature and hydrogen-ion concentration on the stability of the amylases, it should be possible to visualize the actions under conditions different from those customarily present in bread doughs. For example, the high concentration of hydrogen ions resulting during the sponge fermentation of cracker doughs (Johnson and Bailey, 1924) should result in the reduction of cereal alpha-amylase activity to such a degree that very little would be effective when the dough goes to the oven. The neutralization of the sponge brings the dough to a hydrogen-ion concentration approximating pH 7.0 or higher. If any alpha-amylase survived the sponge, this concentration would be conducive to the retention of activity during baking. However, levels of pH 7.0 and above are detrimental to the stability of beta-amylase. Accordingly, in the baking of cracker doughs beta-amylase activity should disappear before much action on the gelatinizing starch can be achieved, and it is doubtful if sufficient alpha-amylase can survive the sponge to be operative in dextrinization during the bake. As a consequence, the greater portion of the amylase action on starch occurring in cracker production may be regarded as taking place during the early stages of sponge fermentation when the damaged or available fraction of the flour starch is converted to maltose.

Significance of Amylase Action during Breadmaking. It is readily apparent that a very appreciable degradation of starch occurs during dough fermentation and the baking of bread. The degree of such degradation will depend on the various factors mentioned. The significance of amylase action may be based on the modification of starch properties, on the removal of a portion of this carbohydrate, or on the nature and amount of

the end-products. An adequate answer to the question of which of these results is most important could be given only with a more complete knowledge of the manner in which starch influences baking.

The damaged starch of a fermenting dough has marked water-absorbing capacity. It has been noted earlier that the removal of this fraction by amylase action may effectively reduce the absorption level of a dough. Accordingly, with flours having relatively large proportions of this fraction, it may be necessary to reduce the initial water added for mixing so that optimum dough properties are attained after the rapid removal of available starch. The necessity for adjusting the absorption is particularly important if high levels of amylase supplement are used.

In the baking of bread a certain amount of starch is transformed to products with other properties; the amount of this conversion depends on the level of active amylase (chiefly alpha-amylase) and the rate of temperature rise, particularly through the gelatinization range of the starch. Removal of starch may considerably modify the crumb characteristics of the bread by a decrease in water-holding capacity (Kozmin, 1933). The decrease in viscosity of gelatinized starch, through liquefaction by alpha-amylase, influences the gelling characteristics and at the same time provides a material more easily distributed over the surface of the gas cell. Such liquefaction is believed to have an influence on the crumb characteristics of the finished loaf and, under certain conditions, may influence the size of the loaf.

The influence of the dextrin content of the dough on baking properties is not well understood, but too high a level is known to be detrimental (Kozmin, 1933; Sandstedt, Jolitz, and Blish, 1939; Kent-Jones and Amos, 1940; Freeman and Ford, 1941). The detrimental effect may be partially the result of an excessive reduction in starch content. The properties of dextrins are such that it is conceivable that their nature and content should have an influence on dough properties. It has been postulated by Kneen and Sandstedt (1942) that the amount and nature of the dextrins influence gas retention. Their data indicate that the role of alpha-amylase in modifying loaf volume is more than just a stimulation of gas production. Since the prime function of alpha-amylase is to liquefy and dextrinize starch, it seems probable that any increased gas retention caused by alpha-amylase action may be due to the properties of dextrins and perhaps of liquefied starch. In any event, alpha-amylase supplementation of doughs is coincident with an increase in dextrins (Freeman and Ford, 1941) and usually with enhanced baking value.

The customary concept of the significance of amylase action in doughs is related to the production of fermentable sugars. The importance of this

saccharification depends largely on the level of sugar added to the dough. If sugar is supplied in quantities adequate for yeast action, it is questionable if that provided by starch hydrolysis has any significance whatever. Of course, if the amount of sugar supplement is inadequate, then the production of sugar from starch by amylase action becomes perhaps the most important role of these enzymes. The production of maltose from starch during dough fermentation and again during gelatinization of starch in the oven has been described. In the provision of sugar for fermentation by yeast, the former is the only process of any significance. If a baking formula is such that no sugar and no amylase supplement is added, the production of maltose during fermentation is largely dependent on the amount of available substrate (damaged starch); accordingly, gas production itself is dependent on this substrate. If an amylase supplement is used, the proportion of available starch hydrolyzed to maltose is increased, a more complete conversion is achieved, and an increase in gas production results. The economics of baking may favor the use of sugar as a dough supplement. On the other hand, the maltose produced by the action of the amylases of flour and of amylase supplements provides an economical substrate for yeast fermentation. It is apparent that, whereas the degradation of starch to maltose by amylases takes place whether or not sugar is used in the formula, the significance of this degradation, within certain limits, varies inversely with the amount of sugar added as such.

A discussion of amylase action in doughs would not be complete without mention that other enzymatic degradations of sugar polymers can occur and may have significance. For example, it has been postulated by Baker, Parker, and Mize (1943) that flour pentosans have an important influence on baking properties. They found that the tendency of such pentosans to gel when treated with oxidizing agents was prevented by the action of malt, wheat bran, or wheat germ extracts. It appears that a "disaggregating" enzyme occurs in such materials. These authors attribute the dough-softening characteristic of malt-supplemented doughs, at least in part, to the action of malt on flour pentosans.

In addition to the pentosans it is possible that other sugar polymers may influence baking. The complex of carbohydrate-splitting enzymes present in plants, particularly in malt or other diastatic supplements, may therefore modify dough and bread properties in manners related to, but different from, that characteristic of starch degradation. It appears probable that a part of the starch hydrolysis, the conversion of dextrins to sugars, is influenced by enzymes other than amylases. However, the actions discussed above as characteristic of alpha- and beta-amylase undoubtedly have the most significance even though they may be modified by others as yet little understood.

AMYLASE SUPPLEMENTS

The influence of added amylase on the quality of bread was recognized many years ago. Jago (1886, 1895) had a clear concept of the value of malt supplement. Baker and Hulton (1908) described the use of malt supplement as "common" and postulated that the beneficial effect was due to the action of the "starch-liquefying enzyme" provided by this means. In this regard they supported the finding of Jago (1895) that the important factor was the amylase (diastase) developed during the germination of grain.

The Role of Supplements. Recent studies by Munz and Bailey (1937), Sandstedt, Jolitz, and Blish (1939), Freeman and Ford (1941),

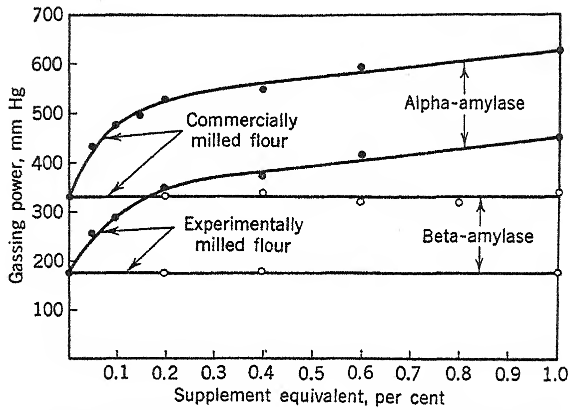


Fig. 2.—Relationship of supplementation with alpha- and beta-amylase to the gassing power of flours (adapted from Kneen and Sandstedt, 1942).

Kneen and Sandstedt (1942), and Meredith, Eva, and Anderson (1944) have established definitely that alpha-amylase is the diastatic component responsible for the increase in dough mobility, the increase in gas production of doughs, and the increase in loaf volume caused by malt supplementation. That this should be true may be seen by referring again to the data in Table I (page 92). Considering the 4-day germination treatment, the free beta-amylase activity was only doubled, whereas the free alpha-amylase activity was increased to a level some three thousand times that of the original wheat. It follows that the addition to flour of even as little as 0.2 per cent of a wheat malt supplement would raise the level of alpha-amylase to an amount several times greater than that originally present, but would have only an insignificant effect on the level of beta-amylase.

The evidence establishing that the response of flour to added malt

depends on the increment of alpha-amylase added is illustrated by Figure 2 and Table III, both of which are taken from Kneen and Sandstedt (1942). Figure 2 shows that the gas production of sugar-deficient doughs is increased by supplementing with alpha-amylase but not by raising the level of beta-amylase. Table III presents data similar to those given by Munz and Bailey (1937), except that the baking formulas carried an excess (6 per cent) of sugar. It would be expected that malt supplementation of dough deficient in sugar would result in increased gas production and therefore in increased loaf volume. It is significant that, even under baking conditions in which sugar was not a limiting factor, increases in loaf volume resulted from alpha-amylase supplementation but not from the addition of malt extracts lacking this component.

TABLE III

RELATIONSHIP OF ALPHA- AND BETA-AMYLASE TO LOAF VOLUME (From Kneen and Sandstedt, 1942)

Malt treatment	Beta-amylase activity	Alpha-amylase activity	Increase in loaf volume	Proof time[1]
	units	*units*	*cc*	*min*
Untreated	8.0	50.0	60	46
Heat treated	0.0	50.0	55	47
Heat treated	0.0	40.0	47	46
Heat treated	0.0	30.0	45	47
Heat treated	0.0	20.0	26	46
Heat treated	0.0	10.0	22	47
Acid treated	12.4	4.8	12	46
Acid treated	6.2	2.4	0	46

[1] "Proof time" is the time required to proof the test loaves to a constant height of 9.5 cm.

The early concept of the role of malt supplement in baking was based principally on the increased sugar production, gas production, and loaf volume obtained by this means. The present reasons underlying the use of such supplements are stated by Freeman and Ford (1941) as: (1) to increase gas production; (2) to improve the crust color; (3) to increase the moistness of the crumb and the keeping quality; and (4) to impart additional flavor. To these may perhaps be added: to increase the gas-retaining ability of the dough.

The manner in which alpha-amylase supplements the action of beta-amylase in the conversion of flour starch to sugar was discussed on page 101. Aside from supplying fermentable carbohydrate for yeast action, the provision of a residue of sugar following fermentation leads to the development of a satisfactory crust color. The characteristic flavor of malt, of course, is not a property of the amylases, but bread flavor may be influenced by the content of sugar and the presence of caramelized sugar in the crust.

The "moistness" of the bread crumb appears to be related to the nature of the carbohydrates present in the finished loaf and may be influenced by starch modification in baking. A relationship of this modification to keeping quality is indicated by reports that the use of malt supplement produces bread with a retarded rate of staling (Bailey, 1932; Schultz and Landis, 1932; Freeman and Ford, 1941).

A further influence on the properties of the gas cell walls is indicated by observations on gas retention. Sherwood and Bailey (1926a) observed that, in addition to increasing gas production in bread doughs, the use of a malt supplement tended to improve the gas-retaining power. Kneen and Sandstedt (1942) concluded from the results of extensive malt supplementation studies with high-sugar formulas that the increases in loaf volume attained could be attributed only to increased gas-retaining capacity. Such increases in gas retention are not the invariable result of alpha-amylase supplementation, but the fact that they do occur indicates that the degradation of starch achieved by this enzyme merits consideration among the factors influencing the baking properties of flour.

The influence of amylase supplementation of flour or dough on bread quality is favorable only up to a certain point. The use of excessive quantities may be extremely detrimental. As early as 1895, Jago noted that very active diastatic supplements must be used with caution, and again, in 1911, Willard and Swanson reported the deleterious effects of wheat germination on the baking strength of the resulting flour. The data of Kozmin (1933) established that the undesirably sticky, moist, and inelastic crumb of bread made from germinated wheat was associated with the enzymatic production of an excess of dextrins. It is evident that a certain degree of conversion of flour starch to dextrins and sugar is desirable in a dough, but also that bread quality is reduced if this conversion is too extensive. From the practical standpoint, the quantity of amylase added must approximate an amount compatible with the production of a desirable loaf of bread.

Sources of Amylase Supplements. It has been indicated above that the value of an amylase supplement for flour is dependent on its alpha-amylase activity. Not only are there several ways in which alpha-amylase may be introduced into flour, but alpha-amylases from different sources also vary in their properties and in their utility.

The introduction of alpha-amylase sufficient to raise the level above that found in normal sound wheat may take place before the grain reaches the mill, during the production and handling of flour, or in the process of breadmaking. Field sprouting of wheat results in a pronounced increase in the alpha-amylase content to levels usually detrimental to baking properties. However, the proper admixture of such field-sprouted wheat

with sound wheat is an economical means of supplementation which is frequently used.

Certain undesirable natural contaminations may lead to increases in flour alpha-amylase. The undesirable features of mold contamination arise largely from the off-flavor imparted and not necessarily from the amylases secreted by their growth. With bacterial contaminations, however, the amylase contributed may be an important consideration. The growth of certain strains of *Bacillus mesentericus* (or *Bacillus subtilis*) is associated with "ropy" bread. These bacteria frequently produce a powerful starch-liquefying amylase which may be a contributory factor in the development of the characteristics of "rope." In general it may be considered that, with the exception of admixtures of small amounts of field-sprouted grain, any introduction of alpha-amylase by natural means is undesirable.

Deliberate alpha-amylase supplementation of flour or dough may be beneficial or not depending on the amount and kind used. Present practices of milling and baking insure close control and avoidance of the introduction of excessive quantities. The choice of the source of alpha-amylase must likewise be governed by consideration of the properties of the enzyme, the nature of the accompanying substances, and compliance with food laws. Alpha-amylase may be obtained from various sources, the most important of which are germinated cereals, fungi, bacteria, and animals.

Germinated cereals have been the most common diastatic supplements. Though barley malt was the first employed and still has considerable popularity, the present trend is toward the use of wheat malt. As early as 1912 Dombach (1912) received a patent covering the addition of germinated wheat to the wheat mixture for flour milling. Swanson (1916) and Olson (1917) observed that the addition of small quantities of germinated wheat to a sound flour led to increases in loaf volume. Sherwood and Bailey (1926, 1926a) made an extensive investigation of the addition of malted wheat to the mill mix, both on laboratory and commercial scales, and demonstrated the practicability of using germinated wheat for mill control of the amylase activity of flour. Present practice is either to mill the malt into flour and introduce this flour into the mill stream or to add increments of whole malted wheat to the grain mix.

The malts of barley and wheat are the only ones used extensively as flour supplements. Rye malt has been used to a minor extent, and there is no obvious reason why the malts of such cereals as oats, sorghum, or maize could not be employed if the addition was in such a manner as to comply with existing food regulations. It has been shown (Kneen, 1944) that all these cereals have the faculty of producing alpha-amylase on germination. The chief difference between them lies in the variation of beta-amylase

content; the malts of barley, wheat, and rye contain abundant supplies of this component, oat malt is low, and the presence of beta-amylase in the malts of sorghum or maize can be detected only by the use of methods designed to evaluate micro quantities. In general, the use of malts should depend on the economics of the production of alpha-amylase. For example, the potential ability of certain sorghum varieties for producing alpha-amylase on germination, coupled with the facility with which this cereal produces alpha-amylase at high malting temperatures (Kneen, 1944), indicates that sorghum might well be used for the preparation of malt supple ment.

Another important group of amylases is that produced by the growth of certain fungi and bacteria. These amylases are generally of the alpha type, and some have been shown to have applicability in flour supplementation, others to be extremely detrimental to bread quality.

Among the molds, certain strains of *Aspergillus oryzae* are particularly adapted to the production of alpha-amylase. The organism is customarily grown on cooked wheat bran; either the crude "mold bran" or the extracted and concentrated enzyme is used commercially, depending on the application. The amylase has starch-degrading properties similar to cereal alpha-amylase. It differs in having less thermostability, the resistance to high temperatures being similar to that of cereal beta-amylase. The use of a purified sterile preparation of mold-amylase concentrate as a flour supplement should give results during dough fermentation similar to those obtained by using malt alpha-amylase. The deficiency in thermostability will be a limiting factor during baking, and starch degradation in the oven should be less pronounced. The data of Green (1934) and of Read and Haas (1936) indicate that mold amylase may be used in baking. The relative economy of production (very high levels of amylase in two or three days) should stimulate further investigation of its utility as a flour supplement.

There is extensive commercial production of an alpha-amylase by the growth of selected strains of *B. subtilis* or *B. mesentericus*. The actions of these amylases appear to be quite comparable with those of other alpha-amylases during dough fermentation.* These enzymes have, however, a much higher degree of thermostability than malt alpha-amylase. Not only is starch breakdown in the oven excessive, but apparently some of the amylase may remain active throughout the baking period and cause liquefaction of the gelatinized starch after the bread is removed from the oven. The use of amylases of such high thermostability produces undesirable bread-crumb characteristics similar to those associated with "ropy" bread.

* Unpublished data obtained in the authors' laboratories.

A group of amylases with starch-degrading properties similar to alpha-amylase, and with thermostability levels similar to or lower than those of the cereal amylases, is that classified as animal amylases. The only one that has much commercial production and utilization is pancreatic amylase. The amylase of "pancreatin" has thermostability of about the same order as mold amylase or as cereal beta-amylase. The optimum hydrogen-ion concentration for its activity is close to *p*H 7.0, that is, a level considerably above that prevailing in bread dough. The data of Green (1934) show that satisfactory results were obtained by the use of pancreatic amylase as a diastatic supplement in breadmaking, and this use has even been patented ("Delta" technische Verkehrs-A.-G., 1933). However, as far as flour supplementation is concerned, the use of animal amylases appears to be impractical. The properties of these enzymes do not indicate that any advantage can be gained, and the economics of production are not favorable to their use.

It thus appears that the commercially feasible amylase agents for flour supplementation are the cereal malts and possibly mold amylases. These are marketed in forms appropriate for their use. Malt may be used as the whole grain, as malt flour, or in the form of malt extracts or malt syrups. Malt flours or malt grains of various levels of activity are the customary mill additions. Bakers may use either the flour, the extract, or the syrup, as individual practice indicates. It should be noted that malt alpha-amylase is markedly unstable in solution and that the alpha-amylase activity of extracts or syrups* frequently shows a reduction with storage. Both malt amylase and mold amylase are available as concentrated dry precipitates with relatively high solubilities in water. These concentrated enzymes of high potency may be diluted with fillers, of which the best for flour supplements appear to be either wheat starch or wheat flour.

SIGNIFICANCE OF DETERMINATIONS OF AMYLASE ACTIVITIES IN FLOUR AND SUPPLEMENTS

Since the amounts and proportions of alpha- and beta-amylase present in a dough may be adjusted rather easily by adding supplements, it has been postulated that the actual amylase content of a flour is not of fundamental importance from the viewpoint of flour quality (Fisher and Halton, 1929; Jørgensen, 1931; Landis, 1934). However, if a flour naturally has an alpha-amylase activity in excess of that considered optimum, amylase content does become a fundamental factor. The occurrence of

* Enzymatically active malt syrups should not be confused with "nondiastatic" syrups designed solely to provide supplemental sugar and flavor.

such flours led Kent-Jones and Amos (1940) to develop a means, the "dextrin figure," for distinguishing them. With all flours the extent of diastatic action in the doughs may influence markedly the quality of the finished loaf. This quality represents the final criterion of the adequacy of modification by amylases. It is therefore pertinent to consider what useful prior information on amylase activity in the baking process can be obtained from analytical determinations made on flour and flour supplements.

Determinations Made on Flours. Certain more rapid measurements have been proposed as useful for assessing the amylase activities of flours. Among these are gas production, autolytic maltose value, the "dextrin figure," and alpha-amylase activity.

Carbon dioxide evolution from flour–water doughs containing yeast has long been considered as indicative of the capacity of flour for gas production during the dough fermentation stage of breadmaking. Conditions simulating dough fermentation are usually approximated with regard to time, temperature, and, to some extent, flour–water–yeast ratios. The test is simplified by the omission of such supplementary ingredients as salt, shortening, sugar, and oxidizing agents. It therefore gives an indication of the inherent capacity of the flour for gas production, and it is dependent on the fundamental carbohydrate–enzyme relations of the flour. Gas production can by no means be considered as an evaluation of amylase activity by itself, since the volume of carbon dioxide evolved is influenced so markedly by starch susceptibility and by the sugar content of the flour. Obviously the technique gives results highly correlated with gas production in sugar-deficient bread doughs and is fundamentally a measure of this property. Such results may or may not be related to the quality of the finished loaf, depending on the amount of sugar available for yeast fermentation. In the United States, the use of high-sugar formulas largely eliminates gas production as a factor influencing loaf volume (Blish and Hughes, 1932) and greatly decreases the practical significance of customary determinations of gas production.

The conditions of the determination of maltose, after autolysis of flour–water suspensions for 1 hour, are even further removed from actual baking conditions. Insufficient time is allowed for the hydrolysis of starch of intermediate degrees of resistance, and the content of nonreducing sugars present in the flour is not recorded. With normal unsupplemented flours, the results essentially record the resistance of the flour starch to enzymatic hydrolysis and, perhaps to a minor extent, the alpha-amylase content of the flour. It has been frequently pointed out that maltose figures do not necessarily indicate the capacity of flour for gas production during dough fermentation (Collatz and Racke, 1925; Pascoe, Gortner, and Sherwood, 1930; Karacsonyi and Bailey, 1930; Blish, Sandstedt, and Astleford, 1932;

Eva, Geddes, and Frisell, 1937; Fisher, Halton, and Hines, 1938; Bottomley, 1938). This is well illustrated by Figure 3 taken from Eva, Geddes, and Frisell (1937). The correlation coefficient between the maltose figure and gas production is not high enough for accurate prediction (+0.751); the scattering of the points shows that 1-hour maltose production, though the major determinant, was only one of the factors influencing 6-hour gas production.

The determinations of gas production and of autolytic maltose production are customarily performed under conditions favorable to the

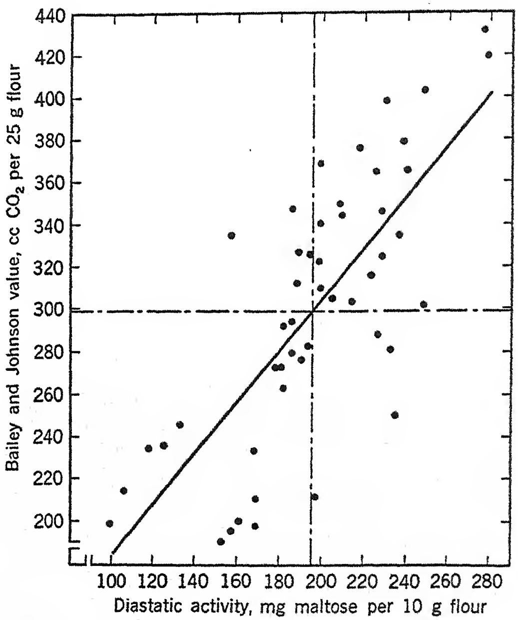


Fig. 3.—Regression of Bailey-Johnson six-hour gas-production values on maltose values (Eva, Geddes, and Frisell, 1937).

actions of both alpha- and beta-amylase. Kent-Jones and Amos (1940) have proposed a method designed to reflect primarily the action of alpha-amylase. Autolysis is carried out at 62°C for 30 minutes and the amount of dextrin produced is evaluated quantitatively and reported as a "dextrin figure." They proposed this method as a control technique designed primarily to differentiate between flours that have satisfactory levels of alpha-amylase and those in which the content of this enzyme is too high to permit the production of a desirable loaf of bread.

The results given by the autolytic methods discussed above may be considered as manifestations of the combined enzyme–substrate system. They do not provide evaluations of the comparative amylase contents of flours. This may be done directly by the extraction of the amylases and determination of their activities under such standard conditions as are required for the specific amylase concerned. The beta-amylase content of flour may be determined by appropriate extraction followed by an evaluation based on its action on gelatinized starch. It is very questionable if such a determination bears any relationship to baking properties, since starch susceptibility rather than enzyme content is the limiting factor in autolytic sugar production by beta-amylase.

The alpha-amylase content of flour may be determined by appropriate extraction followed by evaluation. Customarily, this evaluation is based on the capacity of flour extracts either to liquefy or to dextrinize gelatinized starch. In a previous section of this chapter (page 101), the possible significance of alpha-amylase in dough fermentation and baking has been discussed. It appears, however, that certain general ranges of alpha-amylase content again merit brief mention: that usually occurring in sound, nondiastated flours; the level attained by normal diastatic supplementation; and that found in overdiastated flour or in flour from sprouted wheat. In the first of these cases the level of alpha-amylase may vary from practical absence to one approaching optimum, but usually represents a deficiency. In the second it is important, and economical, to add only sufficient alpha-amylase to achieve the most desirable loaf of bread; accordingly, adjustment should be made with care. In the third case the alpha-amylase content is so high as to be detrimental to baking quality. The degree to which a flour from sprouted wheat must be diluted with sound flour will depend primarily on the alpha-amylase content of the former.

It thus appears that the alpha-amylase content of a flour is an important property. An extension of the concept of Kent-Jones and Amos (1940) therefore is required. Flours may well be divided into three groups depending on their alpha-amylase content: those having a deficiency of alpha-amylase, those having a near optimum content, and those having an excess of this enzyme. From the discussion of supplementation, it will be apparent that the adjustment of a flour to the optimum alpha-amylase content is widely practiced. As yet, the exact relations between flour alpha-amylase and baking properties are not clear, and the desirability of including a determination of this enzyme in routine flour analysis has not been established. Further investigation of possible relations appears to be highly desirable.

Determinations Made on Supplements. The value of diastatic agents

as flour supplements may not be entirely dependent on amylase content. For example, part of the flavor contributed by a malt supplement and the sugar provided by malt syrups may be important. Furthermore, most supplements contain a variety of enzymes in addition to amylase. The possible influence on dough and bread properties of these other enzymes is discussed elsewhere (page 110). The present treatment is limited to consideration of the amylases.

The methods for evaluating amylase supplements are of three types: (1) direct determination of the amylase content; (2) measurement of activity under conditions bearing some similarity to those prevailing during dough fermentation; and (3) evaluation of the influence as reflected in the baked loaf of bread. The last of these is not only the final criterion but also the test upon which other methods of evaluation must be based. Once it is established that a flour with a certain maltose figure or a certain gas production is desirable for a particular baking technique, the necessary adjustment in the level of alpha-amylase can be made. The desire for levels of alpha-amylase higher than those usually found in flours has led to extensive supplementation and to the development of methods for the evaluation of the amylases in supplements.

The fact that the Lintner value of a malt (saccharifying action on gelatinized soluble starch) is not necessarily related to the value of that malt for flour supplementation has been recognized for many years. Nevertheless, this indirect and sometimes misleading evaluation persisted as a means of description, undoubtedly because of the tendency for malts produced under conditions conducive to high saccharifying activity also to have high alpha-amylase development. Many of the early workers apparently were familiar with the increase in autolytic sugar production of flour doughs or suspensions caused by malt supplementation and used this for qualitative evaluation of the diastatic agent. Collatz (1922) contributed a comprehensive investigation of the influence of malt on maltose values, gas production, loaf volume, and bread quality. This work, that of Sherwood and Bailey (1926, 1926a), and other later reports resulted in the trend toward supplementing the flour at the mill rather than, or as well as, in the bakery.

The practice of producing and marketing diastatically fortified flours necessitated the development of a method or methods for establishing the amount of malt to be added. Leatherock, McGhee, and Giertz (1937) proposed a method upon which are based the present techniques used in the mill control of amylase activity. These workers found that, by adding various increments of malt to the same flour and determining autolytic maltose production, the amount of malt necessary to give a desired maltose figure could be calculated. The method served for the

evaluation of malts and was useful for controlling the amount of supplement to be added to a flour. Davis and Tremain (1938) applied the same technique to the evaluation of malt by the gas-production method. A refinement in the gas-production procedure for malt evaluation was proposed by Hildebrand and Geddes (1940). They found that the increase in gas production for any given malt increment was directly proportional to the logarithm of the dosage. This considerably simplified both the calculation of the quantity of a malt required to bring a flour to a desired gas production and the actual evaluation of the malt itself. At the present time then, satisfactory methods are available for use by the miller in the adjustment of the amylase activity of the flour. The same methods serve for the evaluation of malts.

Evaluations of amylase agents based on their effectiveness in baking or in autolytic sugar production of flours necessarily are indirect measures of the effective amylase of malt. Researches culminating in the conclusions of Freeman and Ford (1941) and Kneen and Sandstedt (1942) that the alpha-amylase activity of malt governs its effectiveness as a supplement permit the use of a direct method for amylase determination. No matter whether alpha-amylase is determined directly or indirectly, it must be emphasized that "alpha-amylase supplementation" is a term more apt than "diastatic supplementation" or even "amylase supplementation." Further studies relating alpha-amylase activity to baking behavior, in addition to advancing the knowledge of an important series of reactions in bread doughs, should do much to facilitate a more intelligent use of supplements.

BIBLIOGRAPHY

ALSBERG, C. L., and GRIFFING, E. P. (1925). Effect of fine grinding upon flour. *Cereal Chem.* 2:325–344.

ALWAY, F. J., and HARTZELL, S. (1910). On the strength of wheat flour. *Agr. Expt. Sta. Nebraska, 23rd Ann. Rept.*:100–110.

ANDERSON, J. A., and SALLANS, H. R. (1937). Determination of the diastatic power of malt in degrees Lintner by means of a ferricyanide reagent. *Can. J. Research* C15:70–77.

BAILEY, C. H., and JOHNSON, A. H. (1924). Carbon dioxide diffusion ratio of wheat flour doughs as a measure of fermentation period. *Cereal Chem.* 1:293–304.

—— and MUNZ, E. (1938). The march of expansion and temperature in baking bread. *Cereal Chem.* 15:413–418.

—— and SHERWOOD, R. C. (1923). The march of hydrogen-ion concentration in bread doughs. *Ind. Eng. Chem.* 15:624–627.

BAILEY, L. H. (1932). The use of certain constituents in breadmaking with particular reference to the problem of staling. *Cereal Chem.* 9:65–70.

BAKER, J. C., PARKER, H. K., and MIZE, M. D. (1943). The pentosans of wheat flour. *Cereal Chem.* 20:267–280.

BAKER, J. L., and HULTON, H. F. E. (1908). Considerations affecting the "strength" of wheat flours. *J. Soc. Chem. Ind.* 27:368–376.

BERLINER, E. (1936). Über die Zusammenhänge zwischen Zuckerbildung und Trieb in Weizenmehlteigen. *Mühlenlab.* 6:161–168.

BLISH, M. J., and HUGHES, R. C. (1932). Some effects of varying sugar concentrations in bread dough on fermentation by-products and fermentation tolerance. *Cereal Chem.* 9:331–356.

—— and SANDSTEDT, R. M. (1933). An improved method for the estimation of flour diastatic value. *Cereal Chem.* 10:189–202.

——, ——, and ASTLEFORD, G. R. (1932). Sugars, diastatic activity, and "gassing power" in flour. *Cereal Chem.* 9:378–393.

——, ——, and KNEEN, E. (1938). The cereal amylases with reference to flour and malt behavior. *Cereal Chem.* 15:629–657.

——, ——, and MECHAM, D. K. (1937). Action of wheat amylases on raw wheat starch. *Cereal Chem.* 14:605–628.

BLOM, J., and BAK, A. (1938). Über die Bestimmung von Amylasen durch Verflüssigung von Stärkekleister. *Z. physiol. Chem.* 256:197–207.

——, ——, and BRAAE, B. (1937). α-Amylasen—β-Amylasen. *Z. physiol. Chem.* 250:104–122.

BOTTOMLEY, R. A. (1938). The relationship between diastatic activity (maltose figure) and "gassing power" of experimentally milled flours from some Australian wheats. *Cereal Chem.* 15:509–520.

BROWN, H. T., and HERON, J. (1879). Contributions to the history of starch and its transformations. *J. Chem. Soc.* 35:596–654.

—— and MORRIS, G. H. (1890). Researches on the germination of some of the *Gramineae.* *J. Chem. Soc.* 57:458–528.

CHRZASZCZ, T., and JANICKI, J. (1933). Die wirkliche und scheinbare Amylasemenge in verschiedenen Getreidearten vor und während der Keimung. *Biochem. Z.* 265:260–281.

—— and ——. (1933a). "Sisto-amylase," ein natürlicher Paralysator der Amylase. *Biochem. Z.* 260:354–368.

—— and ——. (1934). The inactivation of animal amylase by plant paralysers and the presence of inactivating substances in solutions of animal amylase. *Biochem. J.* 28:296–304.

COLLATZ, F. A. (1922). Flour strength as influenced by the addition of diastatic ferments. *Am. Inst. Baking Bull.* No. 9.

—— and RACKE, O. C. (1925). Effects of diastase and malt extract in doughs. *Cereal Chem.* 2:213–227.

CORI, G. T., and CORI, C. F. (1940). The kinetics of the enzymatic synthesis of glycogen from glucose-1-phosphate. *J. Biol. Chem.* 135:733–756.

DADSWELL, I. W., and WRAGGE, W. B. (1940). The autolytic digestion of flour in relation to variety and environment. *Cereal Chem.* 17:584–601.

DAVIS, C. F., and TREMAIN, H. E. (1938). A comparison of methods for evaluating the diastatic properties of malt as a flour supplement. *Cereal Chem.* 15:826–831.

—— and WORLEY, D. F. (1934). Correlation between diastatic activity and gassing power in commercial flours. *Cereal Chem.* 11:536–545.

"DELTA" TECHNISCHE VERKEHRS-A.-G. (1933). (Breadmaking.) Fr. Pat. 749,208. July 20. (*Chem. Abstracts* 27:5840.)

DOMBACH, J. G. F. (1912). Process for the treatment of grain. U. S. Pat. 1,040,290.

DÜNNENBERGER, C. (1888). Bakteriologisch-chemische Untersuchung über die beim Aufgehen des Brotteiges wirkenden Ursachen. *Botan. Centr.* 33:245–248, 276–279, 308–311, 341–346, 374–377, 385–395.

ELIZAROVA, S. S. (1940). The β-amylase of wheat and barley. *Compt. rend. acad. sci. U. R. S. S.* 26:698–701.

EVA, W. J., GEDDES, W. F., and FRISELL, B. (1937). A comparative study of various methods of measuring flour gassing power. *Cereal Chem.* 14:458–480.

FISHER, E. A., and HALTON, P. (1929). Relation of hydrogen-ion concentration and buffer value to the baking quality of flour. *Cereal Chem.* **6**:18–33, 97–115, 504–505.

——, ——, and HINES, S. F. (1938). Some observations on maltose production in flour and dough. *Cereal Chem.* **15**:363–377.

FORD, J. S., and GUTHRIE, J. M. (1908). The amylolytic and proteolytic ferments of wheaten flours, and their relation to "baking value." *J. Soc. Chem. Ind.* **27**:389–393.

FREEMAN, H. C., and FORD, W. P. (1941). The evaluation of malt products for use in the breadmaking industry. *J. Soc. Chem. Ind.* **60**:6–11.

GREEN, W. R. (1934). The value of diastatic and other enzymatic activity in flour and doughs. *Cereal Chem.* **11**:319–329.

GREWE, E., and BAILEY, C. H. (1927). Relation of hydrogen-ion concentration of dough to baking properties. *Cereal Chem.* **4**:261–270.

HANES, C. S. (1940). The breakdown and synthesis of starch by an enzyme system from pea seeds. *Proc. Roy. Soc. London* **B128**:421–450.

——. (1940a). The reversible formation of starch from glucose-1-phosphate catalysed by potato phosphorylase. *Proc. Roy. Soc. London* **B129**:174–208.

—— and CATTLE, M. (1938). Starch-iodine coloration as an index of differential degradation by the amylases. *Proc. Roy. Soc. London* **B125**:387–414.

HESSE, A. (1935). Über die Verwendung von Enzymen in der Industrie. II. Teil: Neuere Arbeiten über die Wirkung von Enzymen in der Bäkerei. *Ergeb. Enzymforsch.* **4**:147–172.

HILDEBRAND, F. C., and GEDDES, W. F. (1940). Relations between wheat malt dosage, flour diastatic activity, and gassing power. *Cereal Chem.* **17**:626–635.

HOLLENBECK, C. M., and BLISH, M. J. (1941). Parallelism between starch dextrinizing and liquefying activities of amylases. *Cereal Chem.* **18**:754–771.

HUMPHRIES, A. E., and BIFFEN, R. H. (1907). The improvement of English wheat. *J. Agr. Sci.* **2**:1–16.

——and SIMPSON, A. G. (1909). Gas-making capacity as a factor in the estimation of strength in wheaten flour. *7th Intern. Congr. Pure Applied Chem. London* (abstract in *J. Soc. Chem. Ind.* **28**:738).

JAGO, W. (1886). *The Chemistry of Wheat, Flour, and Bread.* Simpkin, London.

——. (1895). *A Textbook of the Science and Art of Bread-Making.* Simpkin, London.

JØRGENSEN, H. (1931). On the separation of "gassing power" (diastatic activity) from "strength" in baking tests. *Cereal Chem.* **8**:361–374.

JOHNSON, A. H. (1930). Report on diastatic value of flour. *J. Assoc. Official Agr. Chem.* **13**:445–446.

—— and BAILEY, C. H. (1924). A physico-chemical study of cracker dough fermentation. *Cereal Chem.* **1**:327–410.

JONES, C. R. (1940). The production of mechanically damaged starch in milling as a governing factor in the diastatic activity of flour. *Cereal Chem.* **17**:133–169.

JÓZSA, S., and JOHNSTON, W. R. (1935). Determination of alpha-amylase. *Ind. Eng. Chem., Anal. Ed.* **7**:143–146.

KARACSONYI, L. P., and BAILEY, C. H. (1930). Relation of the overgrinding of flour to dough fermentation. *Cereal Chem.* **7**:571–587.

KENT-JONES, D. W., and AMOS, A. J. (1940). A new flour test: the "dextrin figure." *Cereal Chem.* **17**:265–279.

KIRCHHOFF, M. (1816). Formation du sucre. Dans les grains céréales converties en malt, et dans la farine infusée dans l'eau bouillante. *J. pharm.* (sér. 2) **2**: 250–258.

KNEEN, E. (1944). A comparative study of the development of amylases in germinating cereals. *Cereal Chem.* **21**:304–314.

——. (1945). Sorghum amylase. *Cereal Chem.* **22**:112–134.

——, BECKORD, O. C., and SANDSTEDT, R. M. (1941). The starch degrading properties of barley malts. *Cereal Chem.* **18**:741–754.

KNEEN, E., and HADS, H. L. (1945). Effects of variety and environment on the amylases of germinated wheat and barley. *Cereal Chem.* 22:407–418.

——, MILLER, B. S., and SANDSTEDT, R. M. (1942). The influence of temperature on the development of amylase in germinating wheat. *Cereal Chem.* 19:11–27.

—— and SANDSTEDT, R. M. (1941). Beta-amylase activity and its determination in germinated and ungerminated cereals. *Cereal Chem.* 18:237–252.

—— and ——. (1942). The evaluation of malt for use as a flour supplement. *Cereal Chem.* 19:181–195.

—— and ——. (1943). An amylase inhibitor from certain cereals. *J. Am. Chem. Soc.* 65:1247.

——, ——, and HOLLENBECK, C. M. (1943). The differential stability of the malt amylases—separation of the alpha and beta components. *Cereal Chem.* 20: 399–423.

KNYAGINICHEV, M. I., MUTUL, I. F., and PALILOVA, YU. K. (1940). Wheat species characterized according to activity and quality of amylase in their grain. *Compt. rend. acad. sci. U.R.S.S.* 27:1020–1023.

KOZMIN, N. (1933). Biochemical characteristics of dough and bread from sprouted grain. *Cereal Chem.* 10:420–436.

LANDIS, Q. (1934). Diastatic activity in doughs and suspensions. *Cereal Chem.* 11:24–35.

—— and FREY, C. N. (1933). A discussion of the meaning of some terms used in cereal chemistry. *Cereal Chem.* 10:330–346.

—— and —— (1936). The enzymes of flour in relation to yeast fermentation. *Cereal Chem.* 13:281–317.

LAZAR, O. (1938). Some aspects of malting Kaffir corn. *J. S. African Chem. Inst.* 21:23–32.

LEATHEROCK, L. E., McGHEE, H. W., and GIERTZ, J. W. (1937). Diastatic activity in connection with flour mill control. *Cereal Chem.* 14:161–181.

LIEBIG, H. J. v. (1909). Über den Zuckergehalt der feinen Weizenmehle, der Weizenmehlteige und der vergorenen Mehlteige, sowie über die diastatische Kraft der Weizenmehle. *Landw. Jahrb.* 38:251–271.

LINTNER, C. J. (1886). Studien über Diastase. I. *J. prakt. Chem.* 34:378–394.

——. (1887). Studien über Diastase. II. *J. prakt. Chem.* 36:481–498.

—— and ECKHARDT, F. (1890). Studien über Diastase. III. *J. prakt. Chem.* 41: 91–96.

MÄRCKER, M. (1879). Standpunct unserer Kenntnisse der diastatischen Vorgänge. *Landw. Vers.-Sta.* 23:69–70.

MALLOCH, J. G. (1929). Modifications of Rumsey's method for the determination of diastatic activity in flour. *Cereal Chem.* 6:175–181.

——. (1929a). Studies on the resistance of wheat starch to diastatic action. *Can. J. Research* 1:111–147.

MANGELS, C. E. (1926). Factors affecting the diastatic activity of wheat flour. *Cereal Chem.* 3:316–322.

MARKLEY, M. C., and BAILEY, C. H. (1934). Factors affecting the diastatic activity of wheat flour. *Cereal Chem.* 11:515–522.

MAURIZIO, A. (1902). Die Backfähigkeit des Weizens und ihre Bestimmung. *Landw. Jahrb.* 31:179–234.

MAYER, K. (1939). Über die Stärkeverflüssigung. Eine Methode zur Bestimmung der Verflüssigung von Stärkekleister. *Z. physiol. Chem.* 262:29–36.

MEREDITH, W. O. S., EVA, W. J., and ANDERSON, J. A. (1944). Effect of variety and environment on some qualities of malted wheat flour. *Cereal Chem.* 21: 233–240.

MUNZ, E., and BAILEY, C. H. (1937). Effect of the enzymes of malted wheat flour upon certain properties of flour and dough. *Cereal Chem.* 14:445–457.

NEUMANN, M. P., and SALECKER, P. (1908). Über den Einfluss diastasereicher Malzpräparate auf die Backresultate. *Landw. Jahrb.* 37:857–872.

OLSON, G. A. (1917). Wheat and flour investigations. V. *Agr. Expt. Sta. Wash. Bull.* No. 144.

PASCOE, T. A., GORTNER, R. A., and SHERWOOD, R. C. (1930). Some comparisons between commercially and experimentally milled flours. *Cereal Chem.* 7:195–221.

PAYEN, A., and PERSOZ, J. (1833). Mémoire sur la diastase, les principaux produits de ses réactions, et leurs applications aux arts industriels. *Ann. chim. phys.* 53:73–92.

READ, J. W., and HAAS, L. W. (1936). Further studies on the baking quality of flour as affected by certain enzyme actions. *Cereal Chem.* 13:14–37.

RUMSEY, L. A. (1922). The diastatic enzymes of wheat flour and their relation to flour strength. *Am. Inst. Baking Bull.* No. 8.

SANDSTEDT, R. M. (1934). Diastatic activity in suspensions and doughs. *Cereal Chem.* 11:532–535.

——. (1941). The action of the amylases on wheat starch granules. Read at the *Annual Meeting, A.A.C.C.*, May.

—— and BLISH, M. J. (1934). Yeast variability and its control in flour gassing power tests. *Cereal Chem.* 11:368–383.

——, ——, MECHAM, D. K., and BODE, C. E. (1937). Identification and measurement of factors governing diastasis in wheat flour. *Cereal Chem.* 14:17–34.

——, JOLITZ, C. E., and BLISH, M. J. (1939). Starch in relation to some baking properties of flour. *Cereal Chem.* 16:780–792.

——, KNEEN, E., and BLISH, M. J. (1939). A standardized Wohlgemuth procedure for alpha-amylase activity. *Cereal Chem.* 16:712–723.

SCHLEIP, H. (1938). Untersuchungen über die Auswuchsfestigkeit bei Weizen. *Landw. Jahrb.* 86:795–822.

SCHULTZ, A. S., and LANDIS, Q. (1932). The hydrolysis of starch in bread by flour and malt amylase. *Cereal Chem.* 9:305–310.

SHERMAN, H. C., CALDWELL, M. L., and ADAMS, M. (1928). A quantitative comparison of the influence of neutral salts on the activity of pancreatic amylase. *J. Am. Chem. Soc.* 50:2538–2543.

——, ——, and CLEAVELAND, M. (1930). The influence of certain neutral salts upon the activity of malt amylase. *J. Am. Chem. Soc.* 52:2436–2440.

—— and THOMAS, A. W. (1915). Studies on amylases. VIII. The influence of certain acids and salts upon the activity of malt amylase. *J. Am. Chem. Soc.* 37:623–643.

—— and WALKER, F. (1921). The influence of certain amino acids upon the enzymic hydrolysis of starch. *J. Am. Chem. Soc.* 43:2461–2469.

SHERWOOD, R. C., and BAILEY, C. H. (1926). Control of diastatic activity in wheat flour. I. Production of diastatic flour and effect of large dosages. *Cereal Chem.* 3:107–136.

—— and —— (1926a). Control of diastatic activity in wheat flour. II. Experiments with flour milled on a commercial scale. *Cereal Chem.* 3:163–182.

STAMBERG, O. E., and BAILEY, C. H. (1939). Studies on wheat starch. II. The action of amylases on raw wheat starches. *Cereal Chem.* 16:319–330.

STONE, W. E. (1896). The action of enzymic ferments upon starches of different origin. *U. S. Dept. Agr., Off. Expt. Sta. Bull.* 34:29–44.

SWANSON, C. O. (1916). The milling and baking quality and chemical composition of wheat and flour. *Agr. Expt. Sta. Kan. Tech. Bull.* No. 1.

——. (1935). Some factors which affect the diastatic activity in wheat. *Cereal Chem.* 12:89–107.

—— and CALVIN, J. W. (1913). A preliminary study on the conditions which affect the activity of the amylolytic enzymes in wheat flour. *J. Am. Chem. Soc.* 35:1635–1643.

WIJSMAN, H. P., JR. (1890). La diastase considérée comme un mélange de maltase et de dextrinase. *Rec. trav. chim.* 9:1–13.

WILLARD, J. T., and SWANSON, C. O. (1911). Milling tests of wheat and baking tests of flour. *Agr. Expt. Sta. Kan. Bull.* No. 177.

WOOD, T. B. (1907). The chemistry of strength of wheat flour. I. The size of the loaf. *J. Agr. Sci.* 2:139–160.

——. (1907a). A new chemical test for "strength" in wheat flour. *Proc. Cambridge Phil. Soc.* 14:115–118.

ZIEGLER, E. (1940). The effect on diastatic activity of surface, pressure, differential, and temperature of the reduction rolls during milling. *Cereal Chem.* 17:668–679.

CHAPTER IV

ESTERASES

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TERMINOLOGY

The term "esterases" has been commonly used to designate a group of enzymes which catalyze the hydrolysis or synthesis of esters of organic or inorganic acids and alcoholic or phenolic hydroxyl groups. Discussion of esterases here is limited to several of those enzymes active on esters of organic acids. The phosphatases and sulfatases which act on esters or inorganic acids are not included.

Simple Esterases and Lipases

If the specificity of action is largely restricted to an ester substrate of a monocarboxylic acid and a monoatomic aliphatic alcohol, the enzyme is called a simple esterase. It is further characterized by its source in nature, *e.g.*, liver esterase, muscle esterase, etc. Where the alcohol component of the ester is the specific sterol, cholesterol, the enzyme is referred to as cholesterol esterase. Other highly specific esterases are choline esterase, tropine esterase, lecithinases, tannase, and chlorophyllase, each of which receives its name from the substrate upon which it is effective.

The term "lipase" is most frequently associated with those enzymes which are active on glycerides in which the polyhydric alcohol, glycerol, is esterified with fatty acids. Here again, the source of the lipase preparation is frequently used as a prefix, *e.g.*, gastric lipase, pancreatic lipase, castor bean lipase, etc. The terms lipase and esterase have been used interchangeably, principally because of observations that some tissue preparations show activity toward both glycerides and simple ester substrates. Although it is difficult at present to differentiate the action of an esterase and a lipase, it is clearly desirable to use the term "esterase" as the general group

name for all enzymes hydrolyzing esters whether they be simple or complex; to restrict the use of the term "lipase" to those enzyme preparations whose major specificity is toward glyceride substrates; and to assign a specific name to the esterase when the substrate specificity is as clearly shown as it is for some of the azolesterases, such as choline esterase, morphine esterase, and the tropine esterases (Glick, 1942).

In connection with esterase nomenclature, Waldschmidt-Leitz (1929, 1932) has discussed the problem of "relative specificity" of esterases and lipases. Their activity is easily influenced by concomitant and added materials in degrees that vary with different substrates. Although the optimum hydrogen-ion concentrations for esterase and lipase were found to be nearly alike, Amaki (1926) showed a clear differentiation when the effect of a group of alkaloid poisons was studied. Przylecki and Sym (1939) showed that esterases hydrolyze all esters of primary alcohols with equal facility, regardless of the length of the fatty acid chain. Both pancreas and liver preparations are active on carbohydrate esters although the rate of hydrolysis is slow.

Kelsey's studies (1939) particularly emphasize the fact that preparations obtained from a source such as pancreas or liver may contain mixtures of several distinct esterases, although they can be separated only with difficulty. Glick and King (1932) provide an interesting contrast by differentiating pancreatic lipase (lamb) and livere sterase. Acetic precipitations removed the lipase in the precipitate, but most of the esterase remained in the supernatant liquid on similar treatment. Glick and King observed a further difference in that the most active organic inhibitors of the esterase were the best activators for the lipase; these inhibitor compounds also lowered the surface tension in greatest degree. Also, Baker and King (1935) have shown that liver esterase is quite distinct from lecithinase, sulfatase, tannase, phytase, and phosphatases.

Lyo- and Desmolipases

These terms refer to two supposedly different lipases. A small fraction of the lipase in pancreas (about 3 per cent) is apparently somewhat loosely associated with the colloidal matter that comprises the pancreatic cells. This lyolipase is readily extracted with glycerol from the dried pancreatic glands. The remainder of the total lipase activity originally present in fresh pancreatic tissue is called the desmolipase. It is less soluble and is released only after autolysis of the tissue, or after a vigorous crushing operation. As they are prepared, the two enzymes have different properties; this is indicated by their respective activity–*p*S and activity–*p*H curves. Bamann and Salzer (1938) point out that the special properties of the soluble and insoluble lipases can be emphasized by em-

ploying *Ausgleichungs-aktivatoren* (albumin, calcium, chloride, and oleate), which increase the activity of the lyolipase by 5000 to 9000 per cent and that of the desmolipase by only about 100 per cent (Bamann and Laeverenz, 1934).

Prolipase

Rosenheim (1910) obtained an inactive lipase from pancreatic tissue and demonstrated that it was thermolabile and could be activated by serum and tissue extracts. The "coenzyme" activator was thermostable, readily dialyzable, soluble in dilute alcohol, and insoluble in ether. Woodhouse (1932) claimed to have verified the earlier work, showing the activating power of various sera and, in addition, of several inorganic compounds. Rabinowitch and Wynne (1938), however, showed that the activation of washed pancreatic lipase ("prolipase") by either blood sera or the dialyzate from boiled enzyme is largely due to their buffering capacities, a point which was suggested by the work of Umeda (1915) and Fine (1931). A similar "activation" could be observed on adding phosphate buffer or a single amino acid. It is of interest that these authors observed only slight activity of their "prolipase" preparation toward olive oil but marked activity toward triproprionin.

In attempting to repeat the work of Woodhouse, Kelsey found in this laboratory that a pancreatic "prolipase," prepared according to Woodhouse's directions, was already completely activated. The addition of either boiled pancreas or the dialyzate from the original pancreas suspension had no effect on the enzymatic action of the "prolipase." Kelsey suggested that the most likely explanation of the divergent results was that the conditions of reaction he used were sufficient to cause complete activation of the lipase, while in the experiments of Woodhouse the conditions were such as to prevent the maximum amount of hydrolysis unless some activator were included along with the enzyme, *e.g.*, bile salts, calcium, etc.

Blastolipase and Spermatolipase

Lipolytic action has been observed in some plant seeds both before and after germination. In order to differentiate the enzyme in the dormant seed and that in the germinating seed, Willstätter and Waldschmidt-Leitz (1924) designated the former as "spermatolipase" and the latter as "blastolipase." Brand and Sandberg (1926) further describe the properties of these supposedly different enzymes from the same bean.

PREPARATION

The method used to obtain an esterase or lipase depends to a considerable extent on the original source and its lipolytic potency. It has

been a common experience that the properties of the enzyme change markedly during preparation and purification (Willstätter, Haurowitz, and Memmen, 1924). Active preparations of pancreatic lipase may be readily prepared by extracting minced pig pancreas with acetone and ether to dehydrate and defat the tissue. The dried product retains most of its activity for several months on storage in a refrigerator. Either it may be used in this form, or the dried powder can be extracted with water and glycerol (Willstätter and Waldschmidt-Leitz, 1923).

A convenient method of concentrating the pancreatic lipase was developed by Glick and King (1933). They used a 10 per cent solution of sodium chloride to extract the enzyme from the dry pancreatic tissue and found that it could be precipitated quantitatively with magnesium sulfate. Kelsey (unpublished studies from this laboratory) considered it desirable to avoid the use of organic solvents which might denature the protein enzyme. He suggested simply stirring the minced tissue with 2 volumes of 10 per cent sodium chloride for 1 to 3 hours, filtering through gauze, and centrifuging. It was impossible to obtain a clear solution in which the lipolytic activity remained high. Various filter aids such as Celite, infusorial earth, magnesium silicate, alumina, and Permutit were tried at different hydrogen-ion concentration ranges without success. Precipitation of barium sulfate gave a clear solution, but the lipase was quantitatively removed. Dioxane added at 0° caused a precipitation with practically no loss when 5 per cent dioxane was used. Addition of dilute acetic acid (to *p*H 4.6) caused a precipitation of the lipase which could be worked with saline at *p*H 4.6, resuspended in saline at *p*H 8.0, and brought back to *p*H 6.0 without any significant clarification. The same procedure could be carried out by making the suspension half-saturated with ammonium sulfate; again no clarification occurred. Only salt or isoelectric precipitation resulted in an active precipitate. It was observed that Celite at *p*H 8.5 to 9.0, infusorial earth at *p*H 4.6 to 8.0, or Permutit at *p*H 4.6 to 7.0, would permit the lipase to pass through. Unfortunately, however, no large amount of impurities was removed by this treatment. Whenever the solutions were dialyzed, removal of salts irreversibly inactivated the enzyme.

In Willstätter's laboratory much attention has been given to the use of the basic absorbent alumina in the purification of pancreatic lipase. It was found that lipase was more strongly adsorbed than amylase or trypsin which were usually present. Dilute alkali or alkaline phosphate solutions were used to remove the adsorbed lipase.

Working with liver esterases, Baker and King (1935) developed a fairly rapid procedure for obtaining a concentrated preparation of active enzyme. The dried, defatted liver powder was extracted with 0.025 *N* ammonium hydroxide and the nonesterase protein fraction was precipitated

with acetic acid. Other extraneous protein was removed by adding sodium sulfate to 0.4 saturation at 37°, and then the esterase was precipitated by fully saturating the solution in sodium sulfate at *p*H 6.5. The activity, per milligram of total solid, was increased twelvefold by this procedure.

Plant lipases have been similarly prepared. In many instances, a dried, defatted, and sieved seed powder has served as the enzyme without further treatment, although not infrequently an aqueous or glycerol extract has been resorted to (Barton, 1920; Haley and Lyman, 1921). Aqueous extracts are not stable, as Longenecker and Haley (1937) showed with castor bean lipase. Wessel (1941) extracted dried and defatted tung nut meal with a sodium chloride solution to obtain an active esterase solution in which activity was due to both albumin and globulin protein fractions.

Karreth (1939, 1939a) proposed a method for preparing plant lipases free from, or relatively poor in, toxic albumins. An oil emulsion of the lipase is treated with aqueous solutions of organic acids having more than four carbon atoms, or with alkali metal salts of organic acids. It is claimed that the toxic material passes into the aqueous phase. A polyhydric alcohol such as glycerol is then added and the emulsion is centrifuged.

PLANT ESTERASES

Lipases

The first suggestions that lipolytic enzymes existed in plants followed the measurement of increases in acidity in oily seeds when these were crushed or allowed to stand in water. Many years intervened, however, between the first written assertion of such phenomena and the first postulate that it was due to the presence of a "ferment" in the oily seeds and that the increased acidity was due to the increase in free fatty acids. Connstein (1904) mentions that Boussingault showed that the decomposition of such seeds developed free fatty acids. Liebig also noted this action and compared the *fremden Substanzen* to those active in the yeast fermentation of sugars.

Careful quantitative experiments by Pelouze (1855, 1855a) demonstrated an 80 to 90 per cent conversion of the oil in plant seeds to fatty acids and glycerol simply by pressing them to obtain a close contact between the oil and the cell constituents. With less evidence, Schützenberger (1877) postulated the existence of a "ferment." Sachs (1859, 1861) and Peters (1861) recorded the disappearance of oil during the germination of seeds; Fleury (1865) and Muntz (1871) also observed an increase in fatty acids accompanying the oil decomposition in germination.

Green (1891, 1893, 1905) was the first clearly to demonstrate the existence of a lipolytic enzyme. He made his discovery with the castor bean, which has since proved to be a valuable source of a highly active plant

lipase. Green demonstrated that the enzyme is present in the endosperm but not in the embryo and that it occurs as a zymogen, activated by acid. Sigmund (1890) published similar observations independently and almost simultaneously.

Following these early observations, and the classical studies by Büchner (1897, 1898) on the isolation of enzymes that were active when separated from and independent of the cells from which they could be obtained, there were many attempts to locate lipases in other plant seeds. It has been possible to demonstrate lipolytic activity in terms of increased acid production during germination, or after macerating the cells in the presence of water, for a variety of seeds, but the castor bean lipase is the most active of all the plant esterases so far studied. No attempt to prepare a plant lipase equally active with that from the castor bean has yet been successful; nor has it yet been possible to obtain the castor bean enzyme or other plant lipase in crystalline form, as has been the case in the several other types of hydrolytic enzymes.

The hydrolysis of natural fats by castor bean lipase can be accomplished at an extremely rapid rate. For example, Longenecker and Haley (1937) observed 40 per cent hydrolysis of glyceride in 10 minutes, and it was not unusual to effect 80 to 90 per cent hydrolysis in 30 to 40 minutes.

The high toxicity of lipolytically active castor bean preparations has made it desirable to locate other sources of equally active lipases. While other seeds have provided a partial answer, promising results have been obtained by Kirsh (1935, 1935a), who has found an active lipase in the microorganisms, *Penicillium oxalicum* and *Aspergillus flavus*, and has indicated methods for producing these organisms for study. Further study of the lipases of microorganisms should prove fruitful.

The lipases of cereal grains have received relatively little attention. Maestrini (1919, 1921, 1921a) has reported on the lipase action of germinated barley, and Van Laer (1921) reported the existence of a lipase in malt extract. Sullivan and Howe (1933) have studied the lipases of wheat. How extensive may be their effect is not very clearly understood; there is a definite indication here of much-needed further study.

Kretovich *et al.* (1940) observed that the lipase activity of wheat was related to its moisture content. They determined lipase activity by titration of the aciditity of ether extracts of 5 g of ground wheat incubated for varying periods of time. Other studies on the respiratory metabolism and enzymatic activity of the wheat kernel during ripening have been carried out by Smirnov *et al.* (1943).

Recently, Olcott and Fontaine (1941) investigated cottonseed lipase. Dormant cottonseeds contained no active enzyme, but on germination the enzyme appeared to develop. Johnston and Sell (1944) have found a

marked increase in lipase activity of tung kernels on germination. The maximum activity was reached between 16 and 23 days after planting the seed. Bhide and Sahasrabuddhe (1943) have also reported similar studies on germinating seeds.

Bamann and Ullmann (1942) studied the lipases of many higher plants. Most of these enzymes were active at *p*H 8.5 to 10.5 but possessed only weak activity in acid media. This behavior is in marked contrast with that of the lipase of ungerminated castor seeds which has optimum activity at *p*H. 4.7. Unripe castor seeds had a lipase active at *p*H 8.5 to 10.5. Interestingly enough, these authors could find no relationship between the oil content and lipase activity of seeds, and only a slight correlation between lipase activity of the seed and of the vegetative tissue of the same plant.

Chlorophyllase

From time to time there have been studies and reports of plant esterases highly specific in their action on esters peculiarly of plant origin. Thus Willstätter and Stoll (1910, 1911) have found chlorophyllase in the green leaves of all classes of plants investigated. The enzyme action is restricted to chlorophyll itself and its derivative, pheophytin; the ester linkage of one of the three carboxyls of the chlorophyllide with phytol is cleaved, and the reaction may be followed by a simple colorimetric measurement based on the free chlorophyllide formed.

Tannase

Tannase appears to have a special affinity for the esters of phenol carboxylic acids. The enzyme has been found in *Aspergillus niger* by Fernbach (1900) and Pottevin (1900) and has been used with considerable success by Freudenberg (1919, 1921, 1922) in the elucidation of the structure of natural tanning materials. Dyckerhoff and Armbruster (1933) differentiated clearly between ordinary mold esterase and tannase by showing that tannase splits only those esters whose acid component contains at least two phenolic hydroxyl groups in the molecule, neither of which can be ortho to the carboxyl group. Tatarskaya (1936) has discussed the preparation and determination of an active tannase.

Phosphatases and Sulfatases

These enzymes are also widely distributed in both the plant and animal kingdoms. Their differentiation from ordinary esterases is clear in some cases but has not yet been established for the entire group. Current progress in the study of these important enzymes will inevitably clarify the

confusion which now attends the consideration of much of the reported literature concerning their action as esterases.

ANIMAL ESTERASES

The most studied, if not the most important, animal esterase is the lipase of the pancreas. Sumner and Somers (1943) state that, in 1834, Eberle observed the action of pancreatic juice on the neutral fat. Bernard (1849) commented on the lipolytic action of the pancreatic juice. The pancreatic lipase was studied extensively by Willstätter and his colleagues. It was on the basis of their adsorption studies, largely with this enzyme, that the Willstätter *Trager* theory was advanced for the nature of enzmyes (*cf.* Waldschmidt-Leitz, 1929).

Although the Willstätter concepts precluded the consideration of enzymes as proteins, Glick and King (1933) effected a considerable purification of pancreatic lipase and also liver esterase by treating them as proteins. They found solubility characteristics of a globulin for the most active fractions of the pancreatic lipase, which led Kelsey in more recent work in this laboratory to attempt an isolation by protein crystallization techniques. Although unsuccessful in the primary objective, the study emphasized again the protein nature of the active enzyme. Bamann and Laeverenz (1934) reported the isolation of a "crystalline lipase protein"; but, as Sumner (1935) pointed out shortly thereafter, they "do not present evidence which would enable one to consider how this is related to the lipase."

It is inevitable that crude preparations will continue to be found with both simple esterase and lipase activity (Lo Monaco, 1930; Wolvekamp, 1934; Amaki, 1926). However, it will be of considerable importance to direct future studies to the preparation of highly purified and specific enzymes, irrespective of their physiological significance. Such enzyme preparations will find their use at first in the solution of difficult problems in the structure of naturally occurring lipides and later may well find commercial applications. For example, Kelsey (1939, 1939a, 1939b) has indicated clearly how castor bean lipase and pancreatic lipase may be used in lipide analyses. The castor bean enzyme is inactive on cholesterol esters (Reichel and Reinmuth, 1936; Longenecker and Haley, 1937). By taking advantage of this fact, fatty acids combined as glycerides may be separated quantitatively from cholesterol esters occurring in the same sample. Pancreatic lipase preparations contain an active cholesterol esterase. The latter enzyme has the characteristics of a globulin and is therefore difficult to separate from the lipase component. On the other hand, Kelsey (1939a) has shown that the cholesterol esterase activity is destroyed by dilute ammonium hydroxide. Thus the pancreatic preparation can be used in place

of the highly toxic castor bean powder for the same purpose mentioned above.

Kelsey and Longenecker (1941) used a highly specific pancreatic lipase, freed of any cholesterol esterase by Kelsey's procedure (1939), to hydrolyze the glycerides of blood lipides and leave the cholesterol esters intact for further structural studies. This method should find a wide application in the study of the structures of natural lipides. Although the procedure does not permit the separation of the glycerides, it is a simple matter to remove for study the acids formed on hydrolysis. An equally specific sterol esterase would have many applications.

Apparently the specificity of the pancreatic lipase is not so marked that the enzyme action is exerted only upon glycerides of certain fatty acids or certain isomeric glycerides—*e.g.*, symmetrical or unsymmetrical palmitodistearin or optical isomers (Baer and Fischer, 1942; Balls and Matlack, 1938, 1938a; Artom and Zummo, 1937; Weber and King, 1935; Norris, 1941).

Hydrolysis of acid esters of dibasic acids by liver esterase has been studied by Bamann and Rendlen (1936). The effectiveness of the enzyme is increased as the distance between the carboxyl and the ester groups is increased. That the electrochemical nature of the enzyme–substrate compound is determined by the nature of the components was demonstrated by conversion of the carboxyl to an amide, which weakened its electronegative character and increased the affinity of the substrate.

In a study on the mechanism of lipolytic enzyme action, Sobotka and Glick (1934) discuss the fact that "the relative specificity of pancreatic (lipase) for fats and of liver (esterase) for simple esters is the chief point of discrimination between the two enzymes. . . ." They go on to summarize the differences between the two in terms of: (1) physiological function; (2) nature of the characteristic protein fraction; (3) substrate specificity; (4) type of kinetics; (5) differences in affinity for given substrates; and (6) influences of foreign substances on their action.

The existence in muscle tissue of an esterase has been demonstrated by Matlack and Tucker (1940). Lean meat of pork, mutton, beef, and fish contained the enzyme. Quagliarello and Scoz (1932, 1933) had earlier reported the possible existence of a lipase in adipose tissue, a suggestion made by Bottazzi and Schifano (1916) and essentially confirmed by Hepburn and McDuffy (1934).

DIGESTIBILITY STUDIES

Several laboratories have called attention to the use of lipase preparations in studying the relative digestibilities of fats. Dastur and Giri

(1937) and Koch and Duellman (1941) have found the method convenient using either pancreatic lipase or the castor bean enzyme. While there is no doubt that such *in vitro* studies are worth while, it is not yet established that the results will be comparable with actual *in vivo* digestibility. Balls, Matlack, and Tucker (1937–38) point out, for example, that the dependence of the hydrolysis of higher glycerides (*e.g.*, tristearin) on temperature is such that, if behavior at low temperature only were known, pancreatic lipase would have to be regarded as specific for lower glycerides. Glycerides of acids lower than C_8 are split little if any faster at 40° than at 20°, and even at 0° their hydrolysis is remarkably rapid while that of higher saturated fats is practically nil. Olein behaves as though it contains a C_9 rather than a C_{18} acid, possibly because of its *cis* configuration. At 40° the hydrolysis of tristearin or tripalmitin occurs readily despite the fact that the melting points of these compounds are considerably higher.

Tofte (1934) found that hydrogenated oils were not well hydrolyzed by pancreatic lipase. The rate of hydrolysis of oils hydrogenated to varying extents was not directly related to the mean unsaturation (as measured by the iodine value) but to the melting point. If these experiments had their counterpart in animal or human fat digestion, it would be expected that hydrogenated fats would be poorly utilized. Recent unpublished studies in this laboratory have shown clearly, however, that the melting point of a fat may be raised appreciably by a variety of techniques without greatly affecting the digestive coefficient of the fat.

Hartwell (1938) studied this problem by preparing emulsions of a variety of fats in milk and comparing the rate of their digestion by pancreatic lipase to that of a cream prepared similarly from butter. Coconut oil was digested more rapidly than any other fat, and palm kernel oil and castor oil were digested more rapidly than butter. A group of other fats were digested more slowly and at about the same rate: almond oil, peanut oil, bacon fat, beef fat, beef oleo, beef stearin, cocoa butter, cod liver oil, cottonseed oil, lard, mutton fat, olive oil, palm oil, premier jus, soybean oil, hydrogenated whale oil, and hydrogenated peanut oil.

METHODS OF MEASURING ESTERASE ACTIVITY

A variety of methods adaptable to the measurement of either simple esterase or lipase activity have been described. Most of them are dependent upon the acids set free during the catalyzed reaction.

A qualitative demonstration of the presence of lipase can be made by dropping a solution of the test material on plates containing 40 parts of 2 per cent agar, 40 parts of 5 per cent starch, and 1 part of any fat emulsion

(Carnot and Mauban, 1918). After incubation for 1 hr at 37°C (or 55° to exclude bacterial growth), a saturated solution of copper sulfate or copper acetate is dropped on the area containing the enzyme. After 5 to 10 minutes, a blue-green color characteristic of the copper salts of fatty acids appears if lipolysis has taken place.

For quantitative measurement, direct titration of the liberated acids has been found suitable when proper precautions are observed. However, emulsification of the reaction mixture, control of hydrogen-ion concentration throughout the reaction, effect of the buffer, use of enzyme preparations free from insoluble impurities, and end-product removal, are important considerations which have not always been taken into account. For example, Ono (1939, 1939a) has found that the action of either pancreas or *Ricinus* lipase is parallel to the emulsifying power of the fat and the added substances and that the *Ricinus* lipase action is retarded especially by higher unsaturated fatty acids.

The enzyme preparation is added to a substrate (*e.g.*, olive oil for a lipase determination) and after a definite incubation period ethyl alcohol and ether are added to the digest to stop the reaction and the free acids are titrated with standard alkali using phenolphthalein or bromothymol blue as the indicator. Willstätter *et al.* (1923); *cf.* also Waldschmidt-Leitz and Junowicz, 1934) introduced a standardized procedure for determining the relative activity of different pancreatic lipases. One lipase unit was defined as that quantity of a dried preparation which would hydrolyze 24 per cent of 2.5 g of olive oil (saponification number 185.5) in 1 hr at 30°, in the presence of 2 ml of N ammonia–ammonium chloride buffer (pH 8.9), with 10 mg of calcium chloride (0.5 ml of a 2 per cent solution) and 15 mg of albumin (0.5 ml of a 3 per cent solution), and in a total volume of 13 ml, including the volume of the enzyme solution. The reagents were mixed in the order indicated and the time of addition of calcium chloride noted. After addition of the albumin solution, the mixture was shaken vigorously for 3 minutes. The reaction was stopped after definite time intervals, and enough neutral 95 per cent alcohol was added to make a total volume of about 125 ml. Ether (20 ml) and 12 drops of 1 per cent thymolphthalein were added and the mixture titrated to a deep blue with 0.2 N alcoholic sodium hydroxide.

For a good pancreas preparation, 10 mg of the dried gland was found to have 1 lipase unit. In order to relate the activity of a series of preparations, the term "lipase value" was adopted; it represents the number of lipase units in 10 mg of total solids. For example, under these conditions, 6.25 mg of a particular preparation gave 15.1 per cent hydrolysis ($\approx$0.52 unit). On the basis of the original (10 mg $\approx$ 24 per cent hydrolysis = 1 unit) the lipase value of the unknown can be calculated: 0.52 $\times$

(10/6.25) = 0.83. By the use of absorption purification techniques, two with alumina and one with kaolin, Willstätter *et al.* were able to raise the activity of this preparation to a lipase value of 207, a purification of 256 times.

Glick and King (1933), starting with a sheep pancreatic lipase of lower initial activity (lipase value = 0.15), were able to obtain a small amount of globulin fraction (soluble in 10 per cent sodium chloride and twice precipitated with magnesium sulfate) with a lipase value of 134.1, representing a purification of 894 times. The latter workers point out, however, that the use of lipase values alone gives rise to erroneous interpretations regarding the actual extent of purification. They prefer to express the enzyme activity in terms of lipase units per milligrams of nitrogen, a procedure suggested earlier by Sherman *et al.* (1926) for use in following the purification of pancreatic amylase. On this basis, the sheep pancreatic lipase was concentrated only 10.7 times instead of 894 times.

The hydrogen-ion concentration of the Willstätter digest changes gradually until, at 24 per cent hydrolysis, it becomes pH 5.5 to 6.0. Also, the emulsification is not as complete as is desirable. Balls, Matlack, and Tucker (1937–38) surmounted these difficulties by a method that has subsequently been used in other investigations. To approximately 0.00565 mole (0.5 g) of olive oil, or other fat substrate, in a 125-ml glass-stoppered bottle, 5 ml of a solution of bile in glycerol, and glass beads are added. After the oil has dissolved, there are added 10 ml of 0.05 N ammonia–ammonium chloride (pH 8.0), 100 mg of calcium chloride in water, and 0.25 ml of 3 per cent phenolphthalein. The total volume is adjusted to 30 ml after addition of the enzyme solution. The reaction is carried out at constant temperature and small amounts of ammonia are added as a change in color indicates developing acidity. At intervals, an aliquot of 5 ml is withdrawn into 75 ml of neutral mixed solvents (9 alcohol:1 ether) for titration with standard alcoholic potassium hydroxide. Titration at x minutes is corrected for the blank at 0 minutes in order to calculate the quantity of fatty acid set free.

In the final titration, alkali is consumed by free fatty acids liberated by enzyme action, by free fatty acids in the original oil, by the buffer, and by the enzyme itself. Since it is desired to know only the liberated fatty acids, a blank must be included to correct for titration of the other three factors. In addition, the titration value which would result from complete hydrolysis of the oil must be known. This value can be readily obtained by dividing the saponification number of the oil substrate by the ratio $5.61/2.5 = 2.244$; where 5.61 is the number of milligrams of potassium hydroxide equivalent to 1 ml of a 0.1 N solution, and 2.5 is the weight of oil used in the determination. For olive oil with a saponification number of

18.5, there would be required 185.5/2.244 = 82.7 ml of 0.1 N alkali. Longenecker and Haley (1937) express the per cent hydrolysis as follows:

$$100\,\frac{\text{ml } 0.1\ N \text{ alkali (sample)} - \text{ml } 0.1\ N \text{ alkali (blank)}}{\text{saponification number} - \text{free fatty acid number}} = \text{per cent hydrolysis}$$

The saponification number and the free fatty acid number are both expressed in ml 0.1 N alkali.

An alternate expression of lipase activity, which the author found very useful in working with highly active castor bean lipase preparation (Longenecker and Haley, 1937), was made in terms of the time T required to effect a 40 per cent hydrolysis of olive oil, and the weight W of the dried preparation in grams. A formula was arbitrarily adopted as a basis for evaluating various preparations:

$$1 \text{ lipase unit (L.U.)} = 1000/WT$$

The time for a 40 per cent hydrolysis could best be determined by plotting the rate of the reaction and reading from the graph the time at which 40 per cent hydrolysis would have occurred. Thus, one preparation used in a standardized determination in varying amounts, 0.06, 0.07, 0.08, and 0.10 g, required 26, 23, 19.5, and 16 min, for 40 per cent hydrolysis, and the calculated activities were 641, 621, 636, and 625 units, respectively. The same sample ground more finely and tried again in varying amounts, 0.05, 0.06, 0.08, and 0.10 g, required 22, 19.5, 15, and 11 min, for 40 per cent hydrolysis, and the calculated activities were 910, 900, 890, 909 units, respectively. In selecting 40 per cent hydrolysis, consideration was given to the fact that no retardation of the enzyme action due to accumulation of end-products and contact with the water in the digestion mixture was noticed until some time after 50 to 60 per cent of the substrate had been hydrolyzed.

The use of buffers to maintain a constant hydrogen-ion concentration during the reaction, although desirable in general, has some bad features, as Sobotka and Glick (1934a) have demonstrated by activity–pH curves for human liver esterase with buffers containing diammonium phosphate.

Knaffl-Lenz (1922, 1923), Stedman *et al.* (1932), and Harrer and King (1941) greatly improved the technique of esterase measurements by employing continuous titration of the liberated acids with a standard base in a buffer-free medium. The reaction is carried out with 2 ml of a saturated aqueous solution of ethyl butyrate, 0.25 ml of 0.1 per cent bromothymol blue, and water to give a total volume of 8.0 ml including the enzyme solution. The hydrogen-ion concentration is kept constant by addition of 0.01 N sodium hydroxide over a 1-hr period. Esterase units are expressed as ml 0.01 N alkali used in 30-min reaction time per mg of dry weight of en-

zyme. Even here, however, the indicators used may exert an effect on the enzyme system, as Bamann and Schmeller (1931) reported for liver esterase.

An excellent micro method for determining esterase activity has been described by Glick (1934). Methyl butyrate is buffered with glycocoll–sodium hydroxide at pH 8.7, and the digest is titrated to pH 6.5 with 0.05 N hydrochloric acid in the presence of phenol with bromothymol blue as indicator.

Continuous titration with standard alkali of the free acid produced during enzymatic hydrolysis was used by Rona and Ammon (1927) with electrometric rather than indicator methods to indicate hydrogen-ion concentration changes. Glick (1937), Lenti (1939) and, later, Wessel (1941) devised an experimental apparatus employing a glass electrode and calomel half-cell without a buffer in conjunction with equipment for reading hydrogen-ion concentration continuously. Liberated acids were neutralized by the addition of 0.025 N alkali as needed to maintain the desired hydrogen-ion concentration within ± 0.02 unit. In the blank determination, a heat-inactivated enzyme extract was used. Wessel reported difficulty in adapting the method to water-insoluble substrates, but Lucey (1942), working in the same laboratory, was later able to overcome this factor.

The objectionable feature of dilution by continuous titration was clearly recognized by both Wessel and Lucey. Lucey points out that the digest may be increased in volume by as much as 25 per cent. There is involved a continual change in both enzyme and substrate concentration. Lucey practically eliminated such dilution effects by turning to the use of a more concentrated alkali solution (1 N) delivered from a microburette which permitted readings to 0.0002 ml.

The measurement of esterase or lipase activity need not necessarily be confined to a titration of the liberated acids. Nicolai (1926) and Rona and Lasnitzki (1924), for example, used a gasometric method in which the carbon dioxide set free from Ringer's solution by the liberated acids was measured manometrically. Stedman and Stedman (1935) adopted this procedure with excellent results to the Barcroft differential apparatus instead of the Warburg. The method has limitations which have been frankly admitted, but it does offer possibilities for further study.

A turbidimetric method for studying lipase action was proposed by Gózony *et al.* (1929). The method is based upon the precipitation of casein dissolved in sodium hydroxide by acids liberated from an aqueous butyrin solution. The amount of turbidity is dependent on the amount of casein thrown out of solution. This method may prove helpful in some cases but would seem to need considerable study before it could be used in quantitative work.

Rona and Michaelis (1911) presented a stalagmometric method,

later modified by Willstätter and Memmen (1923), for lipase determination with tributyrin as a substrate. The method is based on the measurement of the variation in surface tension produced by a preparation under the following conditions: a total volume of 60 ml containing 56 ml of saturated tributyrin solution, 2 ml of *N* ammonia–ammonium chloride buffer (*p*H 8.6), and, as activators, 10 mg of calcium chloride, 10 mg of sodium oleate, and 30 mg of albumin.

Dilatometry has also been employed in lipase studies. Rona and Ammon (1932) outlined a method based on volume changes during fat hydrolysis which appears to have more possibilities than are indicated by its meager use. A new type of dilatometer was introduced by Ammon and Bartscht (1934) for the study of ester formation and hydrolysis.

Nephelometry was employed by Rona and Kleinmann (1924, 1925, 1926), and Herzfeld (1934) proposed a simple method for determining lipase action based on the Tyndall effect.

It is quite apparent from the above discussion of methods that most of the workers in this field have concentrated their attention on only one of the end-products of the esterase action, the fatty acids. For many purposes this has been entirely satisfactory. It is not to be expected *a priori*, however, that glycerol determinations at varying stages of the reaction will show the release of an amount of glycerol corresponding exactly to the liberated fatty acid. Expressed another way, it is not to be expected that all the glyceride molecules attacked are immediately split to glycerol and three fatty acids. A more rational view of the reaction is that it proceeds in stages in which one acid group is split off from the triglyceride at a time with the resulting intermediate production of di- and monoglycerides. Several years ago the author obtained indirect evidence that such was the actual course of the enzymatic hydrolysis of olive oil, soybean oil, and several animal fats. Although the work was interrupted before completion, sufficient progress had been made to indicate clearly, by glycerol measurements, acetyl values, and direct isolation of mono- and diglyceride intermediates, that these products are formed as intermediates. Virtanen and Lindeberg (1936) reported a similar finding in the pancreatic splitting of tributyrin.

SOME PHYSICAL AND CHEMICAL FACTORS AFFECTING ESTERASE ACTIVITY

Temperature. Balls, Matlack, and Tucker (1937–38) and Balls and Tucker (1938) point out the interesting fact that fat-splitting enzymes remain active at quite low temperatures (−15 to −30°C). Olive oil and tributyrin were hydrolyzed at measurable velocity despite the fact that at these temperatures the system was solid. A sudden change in the rate of

hydrolysis was observed as a change in physical state occurred. Hence it is reasonable to assume that enzymatic fat deterioration is possible in frozen foods.

Stearn (1938), Lineweaver (1939), and Sizer (1943) have discussed at length the theoretical aspects presented by such data as those just mentioned. Lineweaver reaches the important conclusion that the temperature characteristics of organized biological processes have a formal relation to the energies of activation of enzymatically and nonenzymatically catalyzed reactions. In cold-storage temperature conditions, the unorganized enzyme reactions are enhanced relative to the microbial action and nonenzymatically catalyzed reactions. Cold storage may be expected to protect most of those materials whose enzymes have high activation energies. More recently, Lineweaver (1944) has discussed enzyme activity with relation to vegetable dehydration.

Optical Specificity. Dakin (1904, 1905) first showed that lipases do not hydrolyze the two components of a racemic mixture with equal velocity. Liver esterase hydrolyzed *d*-ethyl mandelate preferentially. Some additional studies with racemic mixtures were reported by Willstätter and Memmen (1924) and Willstätter, Haurowitz, and Memmen (1924) with pancreatic and gastric lipase. Rona and Ammon (1927) used the pure optically active forms and found that liver esterase actually hydrolyzed the *l*-form with greater velocity than the *d*-form although the latter was selected from the racemic mixture. Willstätter, Kuhn, and Bamann (1928) explained this phenomenon on the basis of the Michaelis–Menten theory (1913); and later Weber and Ammon (1928) confirmed the fact that the affinity constant (K_m) obtained from observations on the racemic mixture was equal to that obtained by multiplying the values of the two constants measured separately on the *d*- and *l*-isomers. It is obvious, therefore, that observations of "total" enzyme specificity toward a racemic mixture are not adequate for comparisons between related series of compounds.

In studying the optical specificity of liver esterase and pancreatic lipase, Bamann and Feichtner (1936) introduced the consideration of an inactive carrier (pheron) and an active group (agon) which form a complex. Whereas the carriers from pancreas and liver were quite different, the active groups appeared to be the same. However, a synthetic "liver esterase" produced from liver "pheron" and the "agon" of pancreas failed to show the optical specificity of the natural liver esterase. It was suggested that the manner in which the active group is attached to the colloidal carrier ultimately determines the stereoisomerism of the synthetic "enzyme."

When pancreatic lipase and liver esterase are used simultaneously, the hydrolysis of the racemic mandelic ester is much slower than that noted when either one is used alone (Bamann, Feichtner, and Salzer, 1936). Also,

the optical behavior of the isolated mandelic acid is different from the expected. Liver esterase is apparently nonspecific in its action, but the pancreatic lipase acts preferentially on one component of the racemate and shows an autocatalytic effect in the course of its action.

It is of considerable interest to note that, on the basis of present evidence, pancreatic lipase has no specificity toward optically active glycerides. Neither Norris (1941) nor Baer and Fischer (1942) observed a differential attack on highly purified synthetic glycerides.

Inhibition. It is clearly recognized that enzymes may form reversible compounds not only with the substrate but also with the end-products of the reaction and with certain other types of quite unrelated compounds. The retardation of enzyme reaction as a result of "competitive inhibition," with the formation of enzyme–inhibitor complexes, has interesting practical significance in the search for antibiotic substances.

In a system comprising the lipase, a normal substrate, and a third unrelated material, it is easily possible to measure the extent of formation of lipase–substrate and lipase–inhibitor compounds by measuring the extent to which the lipolysis is inhibited. Of considerable value in a study of this sort is the information provided on the nature of the enzyme itself. Glick and King (1931) expressed it thus, " . . . it would be reasonable to assume the presence of groups in the enzyme which would both absorb (by physical attraction forces) and combine with (chemical reaction) suitable substrates or inhibitors."

The following is a partial list of the varied types of compounds which have been shown to combine with esterases and lipases, presumably by the formation of enzyme–inhibitor complexes which effectively reduce the enzyme concentration available for combination with the substrate:

Alkaloids (Rona and Pavlović, 1922–23)
Chloroform, iodoform, and formaldehyde (Palmer, 1922)
Ketocarboxylic acid esters (Willstätter, Kühn, Lind, and Memmen, 1927)
Ketones and aldehydes (Murray, 1929, 1933; Weinstein and Wynne, 1936)
Secondary alcohols (Murray and King, 1930; Glick and King, 1931)
Primary alcohols (Glick and King, 1931)
Hexylresorcinol (Glick and King, 1932, 1932a)
Lyotropic series of radicals (Glick and King, 1932; Weinstein and Wynne, 1936)
Halogen derivatives of acetic acid (Weinstein and Wynne, 1936)
Chloromercuribenzoic acid and organic arsenic compounds (Barron and Singer, 1943)
Salts of fatty acids (Weber and King, 1935)

It is possible to draw from these studies certain tentative conclusions regarding the chemical nature of the organic groups which constitute the active centers in the enzyme. In discussing liver esterase, Glick and King (1931) commented that the marked affinity of the esterase for members of a series of substrates or inhibitors with greater length of hydrocarbon chain may indicate the presence of a long-chain hydrocarbon group in the active portion of the enzyme structure. Murray's studies (1929, 1933), and also those of Weinstein and Wynne (1936), indicate that pancreatic lipase contains essential chemical groups which react with the carbonyl group of ketones or aldehydes. Murray and King (1930) remark: "Although the combination almost certainly occurs at the polar carbinol group, the remainder of the molecule plays an important part in the process, and appears to determine the measure of the affinity." Finally, the specificity of esterases for optical isomers, mentioned above, indicates strongly that certain optically active groups exist in the enzyme by which such combinations are made.

Baker and King (1935) point out that all of the inhibitors they studied with liver esterase exhibited " . . . a transition from competitive to noncompetitive type inhibition, with this point of 0.5 maximum velocity being displaced only toward the right at low concentrations and downward at higher concentrations." When they combined inhibitors of similar polarity and similar reactive groups, they observed simple additive inhibitory effects. However, when inhibitors of different polarity and different active groups were combined, the total inhibitory power was distinctly greater than an additive effect. Baker and King interpret these results as strong evidence that there are two or more reactive groups in the active enzyme center.

SYNTHETIC ACTION

Several studies have demonstrated that esterases in general, and several specific lipases, could catalyze the reverse of the usual hydrolytic reaction with the resulting production of esters. Kastle and Loevenhart (1900) first observed such synthesis on a limited scale, and it was further studied in detail by a series of investigators (Armstrong and Gosney, 1914; *cf.* Ammon, 1940). Liver esterase catalyzes the synthesis of esters of acids from acetic to butyric but shows little action with higher fatty acids. Pancreatic lipase, on the other hand, easily esterified 50 to 55 per cent of oleic acid forming largely diolein and a small amount of monoglyceride; when 55 to 65 per cent of the acid was esterified, triolein and diolein resulted (Artom and Reale, 1935, 1936; *cf.* also Groen, 1925, 1926).

Cedrangolo (1938) observed a marked esterification of oleic acid with glycerol catalyzed by dog pancreas. There was less synthetic activity

toward oleic acid and butanol. On the other hand, ox pancreas showed a greater synthetic activity toward oleic acid and butanol than toward oleic acid and glycerol. Both calcium chloride and albumin inhibit the synthetic reactions although they are effective activators for the hydrolytic action of pancreatic lipase. Sodium taurocholate activated the synthetic action, apparently independent of any surface activation since saponin hindered synthesis.

The influence of variations in the composition of the system on enzymatic synthesis of oleic acid and glycerol is emphasized by Benigno (1938). The numerical value of the ratio of glycerol to oleic acid was determined for maximum synthesis. The optimal value of the ratio is dependent upon the original absolute concentration of glycerol and enzyme.

Fabisch (1931) observed a marked difference for geometrical isomers, fumaric (*trans*) esterifying twice as rapidly as maleic (*cis*). The same was not found true, however, for higher fatty acids such as oleic and elaidic or erucic and brassidic.

More recently, Schreiber (1942) observed that, in an optimum range of glycerol concentration (55 to 90 per cent), synthesis of glyceryl esters of oleic acid could be effected to 48 per cent of the calculated possible maximum by 6 of 29 kinds of seeds examined.

Takamiya (1936, 1936a) found that enzymatic synthesis of oils by the castor bean was retarded by oxidation with hydrogen peroxide. He suggests that there may be different optimum redox potentials for hydrolysis and synthesis although the same enzyme is active in each case. In this connection, it may be noted that Ono (1939a) observed that *Ricinus* lipase action was stopped by a small amount of added hydrogen peroxide and that hydrolysis of partially oxidized perilla oil was difficult.

Sym (1936, 1937) used such solvents as chloroform or benzene to disperse organic substrates to facilitate synthesis by animal lipases. He followed the degree of esterification by: (a) alkali titration of the residual acid; (b) hydroxyl titration; or (c) hydrolysis of the ester formed. Both glycerides and cholesterol esters could be synthesized. Velluz *et al.* (1934, 1935, 1939) studied the effect of a variety of organic solvents on ester syntheses by *Ricinus* lipase. Velluz (1928) and Morel and Velluz (1928) had shown that the synthesis follows the general law of mass action.

BIBLIOGRAPHY

AMAKI, J. (1926). Zur Identitätsfrage der Lipase und Esterase. *Tôhoku J. Exptl. Med.* 8:146–173.

AMMON, R. (1940). Die synthetisierende Wirkung der Esterasen. In F. F. Nord and R. Weidenhagen, *Handbuch der Enzymologie,* Vol. I, pp. 367–370. Akadem. Verlagsgesellschaft, Leipzig.

AMMON, R., and BARTSCHT, K. (1934). Die dilatometrische Methode bei Esterbildung und Esterspaltung. *Biochem. Z.* 268:331–335.

ARMSTRONG, H. E., and GOSNEY, H. W. (1914). Studies on enzyme action. XXII. Lipase (IV). The correlation of synthetic and hydrolytic activity. *Proc. Roy. Soc. London* B88:176–189.

ARTOM, C., and REALE, L. (1935). Synthetic products produced by the action of pancreatic lipase on oleic acid and glycerol. *Boll. soc. ital. biol. sper.* 10:884–886. (*Chem. Abstracts* 30:2589.)

—— and —— (1936). Products synthesized by the action of pancreatic lipase on oleic acid and glycerol. *Bull. soc. chim. biol.* 18:959–978. (*Chem. Abstracts* 30:-5599.)

—— and ZUMMO, C. (1937). Digestione pancreatica degli esteri glicerici α,α-dicapril-β-palmitico e α,α-dipalmito-β-caprilico. *Enzymologia* 3:231–234.

BAER, E., and FISCHER, H. O. L. (1942). Synthesis of dimethyl ethers of the two enantiomorphic α-butyrins and their hydrolysis by lipases. *J. Biol. Chem.* 145:61–68.

BAKER, Z., and KING, C. G. (1935). The purification, specificity and inhibition of liver esterase. *J. Am. Chem. Soc.* 57:358–361.

BALLS, A. K., and MATLACK, M. B. (1938). Mode of action of pancreatic lipase. *J. Biol. Chem.* 123:679–686.

—— and —— (1938a). The enzymic hydrolysis of benzyl stearate and benzyl butyrate. *J. Biol. Chem.* 125:539–543.

——, ——, and TUCKER, I. W. (1937–38). The hydrolysis of glycerides by crude pancreas lipase. *J. Biol. Chem.* 122:125–137.

—— and TUCKER, I. W. (1938). Activity of lipase at low temperatures. *Ind. Eng. Chem.* 30:415–416.

BAMANN, E., and FEICHTNER, C. (1936). Die Konfigurations-Spezifität einer "komponierten" Esterase. (Zur Frage nach dem natürlichen Aufbau und der künstlichen Synthese der Enzyme.) X. Mitteilung: Über asymmetrische Esterhydrolyse durch Enzyme. *Biochem. Z.* 288:70–78.

——, ——, and SALZER, W. (1936). Über einen auffallenden Befund bei der gleichzeitigen Einwirkung von Pankreaslipase und Leberesterase (des Menschen) auf einen racemischen Ester. XI. Mitteilung: Über asymmetrische Esterhydrolyse durch Enzyme. *Biochem. Z.* 288:310–314.

—— and LAEVERENZ, P. (1934). Über pankreatische Lyo- und Desmo-lipasen. Vierte Abhandlung: Zur Kenntnis zellgebundener Enzyme der Gewebe und Drüsen in der von R. Willstätter und M. Rohdewald begonnenen Untersuchungsreihe. *Z. physiol. Chem.* 223:1–20.

—— and RENDLEN, E. (1936). Zur Kenntnis der relativen Spezifität der esterspaltenden Enzyme. VI. Mitteilung. Zur Kinetik der Esterhydrolyse durch Enzyme. *Z. physiol. Chem.* 238:133–144.

—— and SALZER, W. (1938). Lyo- und desmo-Enzyme. *Ergeb. Enzymforsch.* 7:28–49.

—— and SCHMELLER, M. (1931). Über den Einfluss der Indicatorfarbstoffe auf das Wirkungsvermögen der Esterasen. Dritte Mitteilung. Zur Kinetik der Esterhydrolyse durch Enzyme. *Z. physiol. Chem.* 194:1–13.

—— and ULLMANN, E. (1942). Untersuchungen über die Lipase höherer Pflanzen. *Biochem. Z.* 312:9–40.

BARRON, E. S. G., and SINGER, T. P. (1943). Enzyme systems containing active sulphydryl groups. The role of glutathione. *Science* 97:356–358.

BARTON, A. W. (1920). The lipolytic activity of the castor and soy bean. *J. Am. Chem. Soc.* 42:620–632.

BENIGNO, P. (1938). Influenza delle variazioni dei componenti del sistema sulla sintesi enzimatica fra acido aleico e glicarolo. *Enzymologia* 5:293–301.

BERNARD, C. (1849). Recherches sur les usages du suc pancréatique dans la digestion. *Compt. rend.* 28:249–253.

BHIDE, N. V., and SAHASRABUDDHE, D. L. (1943). A study of the enzymes present in germinating seeds. *J. Univ. Bombay* A12:81–84.

BOTTAZZI, F., and SCHIFANO, S. (1916). Fat metabolism: II. Existence of a lipase in adipose tissues. *Atti accad. medicochirurgica di Napoli* (*Chemical Abstracts* 11:354).

BRAND, E., and SANDBERG, M. (1926). On the unity of castor lipase. *Proc. Soc. Exptl. Biol. Med.* **23**:541–544.

BÜCHNER, E. (1897). Alkoholische Gärung ohne Hefezellen. *Ber.* **30**:117–124, 1110–1113.

—— (1898). Über zellenfreie Gärung. *Ber.* **31**:568–574.

CARNOT, P., and MAUBAN, H. (1918). Réaction colorée de la stéapsin sur plaques de gélose-graisse-emulsionée par production de savon de cuivre. *Compt. rend. soc. biol.* **81**:98–101.

CEDRANGOLO, F. (1938). Sintesi e scissione enzimatica di esteri organici. *Enzymologia* **5**:1–11.

CONNSTEIN, W. (1904). Über fermentative Fettspaltung. *Ergeb. physiol.* **3**:194–232.

DAKIN, H. D. (1904). The hydrolysis of optically inactive esters by means of enzymes. Part I. The action of lipase upon esters of mandelic acid. The resolution of inactive mandelic acid. *J. Physiol.* **30**:253–263.

—— (1905). The fractional hydrolysis of optically inactive esters by lipase. Part II. *J. Physiol.* **32**:199–206.

DASTUR, N. N., and GIRI, K. V. (1937). Relative digestibilities of edible fats by castor-seed and pancreatic lipases. *Indian J. Med. Research* **25**:427–442.

DYCKERHOFF, H., and ARMBRUSTER, R. (1933). Zur Kenntnis der Tannase *Z. physiol. Chem.* **219**:38–56.

FABISCH, W. (1931). Fermentative Veresterung geometrisch-isomerer Säuren. *Biochem. Z.* **234**:84–100.

FERNBACH, A. (1900). Sur la tannase. *Compt. rend.* **131**:1214–1215.

FINE, J. (1931). On the influence of serum on enzymes, with special reference to its action on trypsin. *Biochem. J.* **25**:647–670.

FLEURY, M. G. (1865). Recherches chimiques sur la germination. *Ann. chim. phys.* **4**:38.

FREUDENBERG, K. (1919). Über Gerbstoffe. I. Hamameli-Tannin. *Ber.* **52**:177–185.

—— and VOLLBRECHT, E. (1921). Zur Kenntnis der Tannase. *Z. physiol. Chem.* **116**:277-292.

—— and —— (1922). Der Gerbstoff der einheimischen Eichen. (10. Mitteilung über Gerbstoff und ähnliche Verbindungen.) *Ber.* **55**:2420–2423.

GLICK, D. (1934). Studies on enzymatic histochemistry. VIII. A micromethod for the determination of lipolytic enzyme activity. *Compt. rend. trav. lab. Carlsberg* **20**:1–5.

—— (1937). Properties of choline esterase in human serum. *Compt. rend. trav. lab. Carlsberg, Sér. Chim.* **21**:225–232.

—— (1942). Hydrolytic enzymes, non-proteolytic. *Ann. Rev. Biochem.* **11**:51–76.

—— and KING, C. G. (1931). Relationships between the structure of saturated aliphatic alcohols and their inhibiting effect upon liver esterase. *J. Biol. Chem.* **94**:497–505.

—— and —— (1932). Relationships between the structure of organic compounds and their inhibiting effect upon liver esterase. Resemblance to a lyotropic series of anions. *J. Biol. Chem.* **95**:477–482.

—— and —— (1932a). Relationships between the activation of pancreatic lipase and the surface effects of the compounds involved. The mechanism of inhibition and activation. *J. Biol. Chem.* **97**:675–684.

—— and —— (1933). The protein nature of enzymes. An investigation on pancreatic lipase. *J. Am. Chem. Soc.* **55**:2445–2449.

GÓZONY, L., GSELL, J., and HOFFENREICH, F. (1929). Ein neues Lipasetitrationsverfahren. *Biochem. Z.* **204**:126–139.

GREEN, J. R. (1891). On the germination of the seed of the castor-oil plant (*Ricinus communis*). *Proc. Roy. Soc. London* 48:370–392.

—— (1893). On vegetable ferments. *Ann. Botany* 7:83–137.

—— and JACKSON, H. (1905). Further observations on the germination of the seeds of the castor oil plant. *Proc. Roy. Soc. London* B77:69–85.

GROEN, J. (1925). Synthetic action of enzymes especially of pancreatic lipase and of intestinal lipase. *Nederland Tijdschr. Geneeskunde* 69, I, 1104–1114. (*Chem. Abstracts* 19:2058.)

—— (1926). The synthetic action of pancreatic and intestinal lipases. *Arch. néerland physiol.* 11:169–183. (*Chem. Abstracts* 21:2485.)

HALEY, D. E., and LYMAN, J. F. (1921). Castor bean lipase, its preparation and some of its properties. *J. Am. Chem. Soc.* 43:2664–2670.

HARRER, C. J., and KING, C. G. (1941). Ascorbic acid deficiency and enzyme activity in guinea pig tissues. *J. Biol. Chem.* 138:111–121.

HARTWELL, G. A. (1938). A note on the digestion of fats by pancreatic lipase. *Biochem. J.* 32:462–466.

HEPBURN, J. S., and McDUFFY, H. (1934). The relative concentration of esterase and lipase in adipose tissue. *Am. J. Pharm.* 106:14–15.

HERZFELD, E. (1934). Über eine einfache Tyndallmetrische Methode zur Messung von Lipasewirkungen. *Mikrochemie* 15:227–232.

JOHNSTON, F. A., JR., and SELL, H. M. (1944). Changes in the chemical composition of tung kernels during germination. *Unpublished.*

KARRETH, A. (1939). Phytolipases. Brit. Pat. 512,567. Sept. 20.

—— (1939a). Phytolipases. Brit. Pat. 512,824. Sept. 26.

KASTLE, J. H., and LOEVENHART, A. S. (1900). Concerning lipase, the fat-splitting enzyme, and the reversibility of its action. *Am. Chem. J.* 24:491–525.

KELSEY, F. E. (1939). The use of lipase in lipid analyses. I. Specificity of the castor bean lipase. *J. Biol. Chem.* 130:187–193.

—— (1939a). The use of lipase in lipid analyses. II. Specificity of pancreatic lipase. *J. Biol. Chem.* 130:195–198.

—— (1939b). The use of lipase in lipid analyses. III. The action of castor bean and pancreatic lipase on blood neutral fat. *J. Biol. Chem.* 130:199–202.

—— and LONGENECKER, H. E. (1941). Distribution and characterization of beef plasma fatty acids. *J. Biol. Chem.* 139:727–740.

KIRSH, D. (1935). Factors influencing the activity of fungus lipase. *J. Biol. Chem.* 108:421–430.

—— (1935a). Lipase production of *Penicillium oxalicum* and *Aspergillus flavus*. *Botan. Gaz.* 97:321–333.

KNAFFL-LENZ, E. (1922). Über die Kinetik der Esterverseifung durch Leberlipase. *Medd. K. Vetenskapsakad. Nobelinst.* 6, No. 3:1–18.

—— (1923). Über die Kinetik der Esterverseifung durch Leberlipase. *Arch. exptl. Path. Pharmakol.* 97:242–261.

KOCH, J. R., and DUELLMAN, SISTER M. D. (1941). A convenient and efficient method for the determination of the digestibility of fats with pancreatic or other lipases. *Oil & Soap* 18:86–88.

KRETOVICH, V. L., SOKOLOVA, A. I., and USHAKOVA, E. N. (1940). The stable moisture content of grain and its effect on the lipase action. *Compt. rend. acad. sci. U.R.S.S.* 27:701–704. (*Chem. Abstracts* 35:8126.)

LENTI, C. (1939). L'azione Esterasica del Benzoilcarbinolo. *Arch. sci. biol. Italy* 25: 254–260.

LINEWEAVER, H. (1939). The energy of activation of enzyme reactions, and their velocity below 0°. *J. Am. Chem. Soc.* 61:403–408.

—— (1944). Testing enzymes in relation to vegetable dehydration. Dehydration Conference 3:7–11. (In *Bibliography of Agriculture* 4 (6), No. 26274, June, 1944.)

LO MONACO, G. (**1930**). Sul problem del'ident. d. lipasi e esterasi. *Boll. soc. ital. biol. sper.* **5**:447–450.

LONGENECKER, H. E., and HALEY, D. E. (**1937**). Further studies on the nature of *Ricinus* lipase and its action. *J. Am. Chem. Soc.* **59**:2156–2159.

LUCEY, SISTER M. R. (**1942**). A study of pancreatic lipase of shark and swine by continuous potentiometric titration at constant pH. Contrib. No. 43 from Biol. Lab., Catholic Univ. of Am., Washington, D. C., 55 pp.

MAESTRINI, D. (**1919**). Enzymes. II. Protease and lipase of germinated barley. *Atti accad. Lincei* **28**:456–458. (*Chem. Abstracts* **14**:2003.)

—— (**1921**). Enzymes of malt, apropos of a note of Marc H. van Laer on "existence of a lipase in the extract of malt." *Compt. rend. soc. biol.* **84**:616–617. (*Chem. Abstracts* **16**:4005.)

—— (**1921a**). Experimental studies on the reaction velocity of plant enzymes. I. Influence of the concentration of enzymes on the reaction velocity of the enzymes of germinated barley. *Arch. farm. sper.* **32**:40–59. (*Chem. Abstracts* **16**:1593.)

MATLACK, M. B., and TUCKER, I. W. (**1940**). An esterase from muscular tissue. *J. Biol. Chem.* **132**:663–667.

MICHAELIS, L., and MENTEN, M. L. (**1913**). Die Kinetik der Invertinwirkung. *Biochem. Z.* **49**:333–369.

MOREL, A., and VELLUZ, L. (**1928**). Contribution à l'étude de la synthèse biochimique glycerides. Sur la réversibilité de l'activité fermentaire du cytoplasme de la graine de Ricin. *Bull. soc. chim. biol.* **10**:478–488.

MUNTZ, M. A. (**1871**). Sur la germination des graines oléagineuses. *Ann. chim. phys.* **22**:472–485.

MURRAY, D. R. P. (**1929**). Molecular constitution and accessibility to enzymes. The effect of various substances on the velocity of hydrolysis by pancreatic lipase *Biochem. J.* **23**:292–308.

—— (**1933**). The activation of papain by cyanide and other substances. I. *Biochem. J.* **27**:543–556.

—— and KING, C. G. (**1930**). The stereochemical specificity of esterases. I. The affinity of liver esterases for optically active alcohols. *Biochem. J.* **24**:190–198.

NICOLAI, H. W. (**1926**). Die Bestimmung der Lipase in keimenden Kiefernsamen. *Biochem. Z.* **174**:373–383.

NORRIS, F. A. (**1941**). The synthesis and hydrolysis of optically active monopalmitin. *Univ. Pittsburgh Bull.* **37**:260–264.

OLCOTT, H. S., and FONTAINE, T. D. (**1941**). Composition of cottonseeds. IV. Lipase of germinated seed. *J. Am. Chem. Soc.* **63**:825–827.

ONO, T. (**1939**). Hydrolysis of fats and fat acid esters. III. Hydrolysis of fats by enzymes. *J. Agr. Chem. Soc. Japan* **15**:1085–1096.

—— (**1939a**). Hydrolysis of fats and fat acid esters. IV. Hydrolysis of fats by enzymes. *J. Agr. Chem. Soc. Japan* **15**:1161–1172.

PALMER, L. S. (**1922**). The influence of various antiseptics on the activity of lipase. *J. Am. Chem. Soc.* **44**:1527–1538.

PELOUZE, M. J. (**1855**). Mémoire sur la saponification des huiles sous l'influence des matières qui les accompagnent dans les graines. *Ann. chim. phys.* **45**:319–327.

—— (**1855a**). Mémoire sur la saponification des huiles sous l'influence des matières qui les accompagnent dans les graines. (Extrait.) *Compt. rend.* **40**:605–611.

PETERS, E. (**1861**). Zur Keimungsgeschichte des Kürbissamens. *Landw. Vers.-Sta.* **3**:1–16.

POTTEVIN, H. (**1900**). Diastase dédoublant l'acide gallotanique. *Compt. rend.* **131**:1215–1217.

PRZYLECKI, ST. J., and SYM, E. A. (**1939**). Über die Wirkung der Pankreas- und Leberesterase auf mehrwertige Alkohole, Kohlenhydrate und ihre Ester. *Enzymologia* **6**:135–139.

QUAGLIARELLO, G., and SCOZ, G. (**1932**). The existence of a lipase in adipose tissues. *Chim. ind. agr. biol.* **8**:408. (*Chem. Abstracts* **27**:1644.)

QUAGLIARELLO, G., and SCOZ, G. (1933). The existence of a lipase in the tissue of adipose organisms. *Arch. ital. biol.* 89:190–200. (*Chem. Abstracts* 28:1371.)

RABINOWITCH, L., and WYNNE, A. M. (1938). Studies on pancreatic lipase. III. The activation of the washed enzyme by blood serum and other substances. *J. Biol. Chem.* 126:109–115.

REICHEL, L., and REINMUTH, W. (1936). Über Wirkungsbedingungen und Spezifität der Ricinuslipase. *Z. physiol. Chem.* 244:78–80.

RONA, P., and AMMON, R. (1927). Zur stereochemischen Spezifität der Lipasen und Beiträge zur Giftwirkung an den fettspaltenden Fermenten. *Biochem. Z.* 181: 49–79.

—— and —— (1932). Versuche über enzymatische Ester-Hydrolyse und -Synthese. *Biochem. Z.* 249:446–454.

—— and KLEINMANN, H. (1924). Nephelometrische Untersuchungen über fermentative Eiweissspaltung. II. Mitteilung: Der Einfluss von Ionen auf die peptische Verdauung. *Biochem. Z.* 150:444–467.

—— and —— (1925). Nephelometrische Untersuchungen über fermentative Eiweissspaltung. III. Mitteilung: Eine Methode zur Bestimmung der peptischen und tryptischen Verdauung von Casein. *Biochem. Z.* 155:34–53.

—— and —— (1926). Untersuchungen über die Beziehung zwischen Substratdispersität und Fermentwirkung mittels einer neuen Methode zur Bestimmung der fermentativen Fettspaltung. *Biochem. Z.* 174:18–42.

—— and LASNITZKI, A. (1924). Eine Methode zur Bestimmung der Lipase in Körperflüssigkeiten und im Gewebe. *Biochem. Z.* 152:504–522.

—— and MICHAELIS, L. (1911). Über Ester -und Fettspaltung im Blute und im Serum. *Biochem. Z.* 31:345–354.

—— and PAVLOVIĆ, R. (1922–23). Über die Wirkung des Chinins und des Atoxyls auf Pankreaslipase. *Biochem. Z.* 134:108–117.

ROSENHEIM, O. (1910). On pancreatic lipase. III. The separation of lipase from its coenzyme. *J. Physiol.* 40:14–16.

SACHS, J. (1859). Über das Auftreten der Stärke bei der Keimung ölhaltiger Saamen. *Botan. Ztg.* 17:177–183, 185–188.

—— (1861). Keimung des Kürbissamens. *Landw. Vers.-Sta.* 3:17.

SCHREIBER, E. (1942). Über den Einfluss der Glycerinkonzentration auf die enzymatische Fettsynthese vermittels Samenlipase. *Z. physiol. Chem.* 276:56–62.

SCHÜTZENBERGER, P. (1877). *On Fermentation.* Appleton, New York.

SHERMAN, H. C., CALDWELL, M. L., and ADAMS, M. (1926). Enzyme purification by adsorption: An investigation of pancreatic amylase. *J. Am. Chem. Soc.* 48:2947–2956.

SIGMUND, W. (1890). Über fettspaltende Fermente im Pflanzenreich. *Monatsh.* 11:272–276.

SIZER, I. W. (1943). Effects of temperature on enzyme kinetics. In *Advances in Enzymology*, Vol. III, pp. 35–62. Interscience, New York.

SMIRNOV, A. I., BRONOVIT͡SKAI͡A, Z. S., PSHENNOVAI͡A, K. V., CHIGIREV, S. D., and USHAKOVAI͡A, E. N. (1943). Dykhatel'nyĭ gazoobmen i fermentnai͡a aktivnost' pshenichnogo zerna pri sozrevaniĭ. [The respiratory metabolism and enzymatic activity of the wheat kernel during ripening.] *Biokhimiya* 8:149–157.

SOBOTKA, H., and GLICK, D. (1934). Lipolytic enzymes. I. Studies on the mechanism of lipolytic enzyme actions. *J. Biol. Chem.* 105:199–219.

—— and —— (1934a). Lipolytic enzymes. II. The influence of hydrogen ion concentration on activity of liver esterase. *J. Biol. Chem.* 105:221–230.

STEARN, A. E. (1938). The theory of absolute reaction rates applied to enzyme catalysis. *Ergeb. Enzymforsch.* 7:1–27.

STEDMAN, E., and STEDMAN, E. (1935). The relative choline-esterase activities of serum and corpuscles from the blood of certain species. *Biochem. J.* 29: 2107–2111.

STEDMAN, E., STEDMAN, E., and EASSON, L. (1932). Choline-esterase. An enzyme present in the blood serum of the horse. *Biochem. J.* 26:2056–2066.

SULLIVAN, B., and HOWE, M. A. (1933). Lipases of wheat. I. *J. Am. Chem. Soc.* 55:320–324.

SUMNER, J. B. (1935). Enzymes. *Ann. Rev. Biochem.* 4:37–58.

—— and SOMERS, G. F. (1943). *Chemistry and Methods of Enzymes.* Academic Press, New York, p. 54.

SYM, E. A. (1936). Action of esterase in the presence of organic solvents. *Biochem. J.* 30:609–617.

—— (1937). The enzymic synthesis of fats and esters. *Polish Agr. Forest Ann.* 40: 406–408. (*Chem. Abstracts* 31:7452.)

TAKAMIYA, E. (1936). Castor-bean lipase and its activator. *Proc. Imp. Acad. Tokyo* 12:73–74. (*Chem. Abstracts* 30:7134.)

—— (1936a). Castor-bean lipase. IX. Enzymic synthesis of oils and its activating substance. *J. Agr. Chem. Soc. Japan* 12:401–405. (*Chem. Abstracts* 30:6771.)

TATARSKAYA, I. (1936). Methods for the determination and isolation of tannase preparations. *Bull. Applied Botany, Genetics, Plant Breeding U.S.S.R., Ser. III* No. 14:117–125.

TOFTE, F. (1934). Versuche über die Spaltung gehärteter Öle durch Pankreaslipase. *Biochem. Z.* 272:308–316. (*Chem. Abstracts* 28:6742.)

UMEDA, N. (1915). Inactive lipase and the nature of its co-enzyme. *Biochem. J.* 9:38–52.

VAN LAER, M. H. (1921). The existence of emulsin and of lipase in malt extract. *Compt. rend. soc. biol.* 84:471–474. (*Chem. Abstracts* 16:1439.)

VELLUZ, L. (1928). Sur la synthèse et l'hydrolyse biochimiques des glycérides. Cinétique des réactions. *Bull. soc. chim. biol.* 10:1213–1226.

—— (1934). Quelques données sur la synthèse des esters par la "lipase" du Ricin. *Bull. soc. chim. biol.* 16:909–916.

—— and LE LOUS, J. (1939). Influence of different organic solvents on the synthesis of esters by Ricinus lipase. *Bull. soc. chim. biol.* 21:814–819. (*Chem. Abstracts* 33:7825.)

—— and SAULEAU, P. (1935). Synthèse biochimique d'esters gras de quelques cyclohexanols. *Compt. rend.* 197:277–279.

VIRTANEN, A. I., and LINDEBERG, E. (1936). Enzymic hydrolysis of tributyrin with pancreatic lipase. *Acta Chem. Fennica* B9:2. (*Chem. Abstracts* 30: 3846.)

WALDSCHMIDT-LEITZ, E. (1929). *Enzyme Actions and Properties.* Translated and extended by Robert P. Walton. Wiley, New York.

—— (1932). Enzymes. *Ann. Rev. Biochem.* 1:69–88.

—— and JUNOWICZ, R. (1934). Zur Bestimmung der Pankreaslipase. *Biochem. Z.* 268:178–180.

WEBER, H. H., and AMMON, R. (1928). Die stereochemische Spezifität von Leber- und Pankreaslipase. *Biochem. Z.* 204:197–207.

WEBER, H. H. R., and KING, C. G. (1935). Specificity and inhibition characteristics of liver esterase and pancreas lipase. *J. Biol. Chem.* 108:131–139.

WEINSTEIN, S. S., and WYNNE, A. M. (1936). Studies on pancreatic lipase. II. Influence of various compounds on the hydrolytic activity. *J. Biol. Chem.* 112: 649–660.

WESSEL, C. J. (1941). A study of the esterase activity of *Aleurites Fordii.* Contrib. No. 38 from Biol. Lab., Catholic Univ. of Am., Washington, D. C., 72 pp.

WILLSTÄTTER, R., HAUROWITZ, F., and MEMMEN, F. (1924). Zur Spezifität der Lipasen aus verschiedenen Organen. Neunte Abhandlung über Pankreasenzyme. *Z. physiol. Chem.* 140:203–222.

—— and KUHN, R. (1923). Über Masseinheiten der Enzyme. *Ber.* 56:509–512.

——, ——, and BAMANN, E. (1928). Über asymmetrische Ester-Hydrolyse durch Enzyme. (I. Mitteilung.) *Ber.* 61:886–895.

WILLSTÄTTER, R., KUHN, R., LIND, O., and MEMMEN, F. (1927). Über Hemmung der Leberesterase durch Ketocarbonsäureester. *Z. physiol. Chem.* **167**:303–309.

—— and MEMMEN, F. (1923). Zur stalagmometrischen Bestimmung der lipatischen Triburyrinhydrolyse. *Z. physiol. Chem.* **129**:1–25.

—— and —— (1924). Vergleich von Leberesterase mit Pankreaslipase; über die steriochemische Spezifität der Lipasen. Achte Abhandlung über Pankreasenzyme. *Z. physiol. Chem.* **138**:216–253.

—— and STOLL, A. (1910). Untersuchungen über Chlorophyll. XI. Über Chlorophyllase. *Ann.* **378**:18–72.

—— and —— (1911). Untersuchungen über Chlorophyll. XIII. Spaltung und Bildung von Chlorophyll. *Ann.* **380**:148–154.

—— and WALDSCHMIDT-LEITZ, E. (1923). Über Pankreaslipase. Zweite Abhandlung über Pankreasenzyme. *Z. physiol. Chem.* **125**:132–198.

—— and —— (1924). Über Ricinuslipase. *Z. physiol. Chem.* **134**:161–223.

——, ——, and MEMMEN, F. (1923). Bestimmung der pankreatischen Fettspaltung. Erste Abhandlung über Pankreasenzyme. *Z. physiol. Chem.* **125**:93–131.

WOLVEKAMP, P., and GRIFFIOEN, K. (1934). Über Lipase- und Esterasewirkung des Pankreassaftes. *Z. physiol. Chem.* **223**:36–42.

WOODHOUSE, D. L. (1932). Investigations in enzyme action directed towards the study of the biochemistry of cancer. The activation of pancreatic prolipase. *Biochem. J.* **26**:1512–1527.

CHAPTER V

ESTERASES IN RELATION TO MILLING AND BAKING

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INTRODUCTION

The esterases of plants have not been investigated as extensively as the amylases and proteolytic enzymes, and therefore a discussion of the function of the esterases as applied to milling and baking problems is necessarily limited. The term "esterases" refers to the entire group of hydrolytic enzymes splitting fats and esters, and hence includes both lipases and simple esterases of phosphoric acid as well as of fatty acids. The terms "lipases" or "lipolytic enzymes" refer to those enzymes cleaving the glycerides of the fatty acids, and the remaining esterases may conveniently be called "simple esterases." It should be emphasized that the terms esterase, lipase, and lipolytic enzymes are often used interchangeably in the literature, and to some extent throughout this chapter, because many enzyme preparations act on both glycerides and simple esters of fatty acids. There are other esterases, such as lecithinase, chlorophyllase, and phosphatases. Of these enzymes, only the phosphatases will be discussed in detail since they are the only esterases on which any studies on wheat have been conducted.

This discussion will be confined largely to the measurement, distribution, and function of the esterases of wheat and the part they play during storage and baking, but, since data in this field are so meager, it will be necessary to borrow freely from research done on other enzyme sources, both plant and animal.

The esterases that are discussed in the first section hydrolyze fats and simple esters of fatty acids when the plant needs to mobilize its reserve of storage fat for the germinating embryo. Conversely, such enzymes can synthesize fats for storage in the new seed just being formed. The lipase

of the castor bean (*Ricinus communis*) has been the most extensively studied of all the plant lipases, but it has not yet been obtained in crystalline form. Most plants probably contain a lipolytic enzyme; its presence has been demonstrated in castor bean, soybean, hemp, flax, poppy, cottonseed, barley, corn, and wheat, among others. Olcott and Fontaine (1941) found that lipase was present in the germinating, but not in the dormant, cottonseed. Perhaps its presence could have been demonstrated by suitable activation of the enzyme in the dormant seed.

There are other esterases present in grains in addition to the lipolytic enzymes and the best known is phytase, an esterase that hydrolyzes phytic acid or its salts to inositol and phosphoric acid. This enzyme, as well as other phosphatases, will be discussed in the second section of this review.

MEASUREMENT OF ESTERASE ACTIVITY

The following reaction is catalyzed by the lipolytic enzymes:

$$RCOOR' + H_2O \rightleftharpoons RCOOH + R'OH$$

If RCOOH is a higher fatty acid and R′OH is glycerol, the catalyst is a lipase; if RCOOH is any other acid and R′OH a monatomic alcohol, the catalyst is a simple esterase.

The most common methods for the measurement of esterases are based on the estimation of the increase in acidity by direct titration under prescribed conditions of time, temperature, and hydrogen-ion concentration. When glycerides act as substrates, glycerol may also be measured, but this technique is seldom employed. A stalagmometric procedure based on changes in surface tension has been used when esters of lower fatty acids serve as substrates. The hydrolysis of the ester causes an increase in surface tension, and this results in a decrease in the number of drops issuing from a dropping pipette or stalagmometer. Manometric determinations have been developed in which the acid formed is measured by the volume of carbon dioxide it liberates from a bicarbonate buffer. In cases in which the lipolytic enzymes exhibit optical specificity, changes in rotation have been utilized as a means of following the hydrolysis of optically active substrates.

Several arbitrary "lipase units" have been proposed, and the percentage of substrate or the number of moles of triglyceride hydrolyzed also serves occasionally as a measurement of activity. More frequently, the quantity of base required to neutralize the acid formed is reported as the index of activity.

Typical Methods. A few typical methods which have been employed in measuring the esterase of wheat are briefly outlined below.

Pett (1935) used the micro apparatus developed by Linderstrøm-

Lang and Holter (1934) to study the activity of the fat-splitting enzymes of the wheat kernel. The method employed by Pett was a slight modification of that described by Glick (1934), in which methyl butyrate is used as a substrate, glycine and sodium hydroxide are used as a buffer, and titrations are made with dilute acid. The butyric acid formed as the methyl butyrate is hydrolyzed is neutralized by the alkaline buffer. The decrease in alkalinity is then directly related to the enzymatic activity. Pett selected *p*H 8.2 in order to obtain suitable titration values, but he stated that this was not the optimum hydrogen-ion concentration. Data for a curve relating hydrogen-ion concentration and enzyme activity could not be obtained with the method used.

The procedure used by Sullivan and Howe (1933) was as follows: Half a gram of finely ground wheat (or other enzyme source) was incubated for 24 hrs at 37.9°C in a 250-ml Erlenmeyer flask with 10 ml of water, 2 ml of toluene, the substrate (usually 1 ml of liquid or 1 g of solid fat), and 10 ml of buffer solution. At the end of the incubation period, 100 ml of 3:1 acetone–ether mixture was added to the sample and also to the blank, which contained all the reagents, substrate, and enzyme, but had been boiled prior to incubation; 0.1 *N* sodium hydroxide was used for titration with 1 per cent phenolphthalein as an indicator. Lipase activity was expressed as milliliters of the sodium hydroxide solution.

Bamann and Ullmann (1942), in studying the lipases of a number of plants, selected the method devised by Willstätter and Memmen (1923). Enzyme activity was reported as butyrase units (B.E.), the amount of enzyme which, in 57 ml saturated tributyrin solution, 2 ml of the selected buffer, and 1 ml of enzyme suspension, causes the number of drops per min from an Ostwald stalagmometer (giving 60 drops per min with pure water) to diminish by 20 at the end of 50 min at 20°C.

Olcott and Fontaine (1941) employed the following modification of the method of Longenecker and Haley (1935) in their investigation of the lipases of the cottonseed: To a weighed sample in a vial were added 1 ml of substrate, 3 ml of water, and 1 ml of buffer. The vial was stoppered, shaken for 16 hrs at room temperature, and the contents were centrifuged. The solid residue was then washed four times by centrifuging with isopropanol–petroleum ether (2:1); the original supernatant layers were combined with the washings and titrated with 0.1 *N* sodium hydroxide in isopropanol using aniline blue as the indicator.

Preparation of the Enzyme. In most of the work on the lipolytic enzymes of plants, the enzyme source has been the material as found in nature or this material defatted by acetone or ether. Fineness of grinding has a significant effect on the activity of the enzyme; the smaller the particle size the greater is the activity. Glycerol extracts have sometimes been em-

ployed in attempts to concentrate the enzyme, but since it is only partially soluble in this, in most other organic solvents, and in water, little has been accomplished on its purification or concentration except for the work of Willstätter and Waldschmidt-Leitz (1924) with *Ricinus* lipase. These investigators prepared an emulsion of the enzyme by centrifuging the extract made by grinding the seeds with water. The "cream" could be further concentrated by adsorption on kieselguhr or by partial removal of protein by means of dilute hydrochloric acid, sodium hydroxide, or salt solution. It was difficult to concentrate the enzyme, and in all processes of purification it was found that the enzyme must be left in contact with the fat; the most successful method involved the preparation of an emulsion of fat and water.

Some progress has been made in the purification of the esterases from animal sources by judicious use of a growing number of methods available for the isolation of proteins, such as fractionation by salt precipitation, by electrophoresis, by ultracentrifugation, and by adsorption techniques. None of these techniques has been utilized for the concentration of the esterases of wheat or other plant materials. Since associated impurities in the enzyme source may considerably modify its properties, there is a fundamental need for such investigations on the plant as well as animal esterases.

Although lipolytic enzymes do not appear to be highly specific, usually either the triglycerides or the simple esters are preferentially hydrolyzed. Lipases may have a selective action on a racemic mixture and hence act at different rates on optical isomers (Dakin, 1904, 1905). Most of the work in this field has been done on the esterases from pancreas or liver. Practically all esterase preparations are mixtures, and methods will undoubtedly be found in the future to separate such mixtures into fractions of greater specificity.

Selection of the Substrate. The nature and concentration of the substrate is of great importance in measurements of esterase activity. Generally, pure lower glycerides or simple esters are used; but frequently, also, emulsified olive oil. Titration values are much less when the substrates are higher glycerides, such as tripalmitin, tristearin, or the fat extracted from wheat. This behavior would be expected because of the lower solubility of the higher glycerides in water. Even when fats of higher molecular weight, such as those from natural sources, are emulsified, the esterase activity, as measured by the amount of fatty acid liberated, is lower than when glycerides possessing greater water miscibility are employed. The ideal substrate to use for a study of the lipolytic enzymes of wheat would be wheat fat, provided that it could be uniformly emulsified by use of some surface-active agent that had no activating or inhibiting effect on the enzyme. Any

natural fat, however, such as that obtained from wheat or wheat germ, suffers from lack of homogeneity since it contains mixed glycerides, phosphatides, sterols, and polyenes. Hence, for a study of enzymatic lipolysis, the most suitable substrate should be a glyceride that is as high in the series as possible, and which is homogeneous and sufficiently water-soluble to allow measurable titration values within a relatively short time interval. Tributyrin appears to be the compromise which meets these requirements most successfully.

Optimum Hydrogen-Ion Concentration. Since *Ricinus* lipase shows its greatest activity at *p*H 4.5–5.0, it has been generally assumed that other plant lipases are active in this range. It was reported by Sullivan and Howe (1933), however, that the optimum range for wheat lipase, acting on triacetin, is on the alkaline side, *p*H 7.2–8.2. Triacetin was used in this investigation because it gave the highest titration values of any substrate. Tributyrin would have been a better choice for the reasons mentioned in the discussion on the selection of a substrate. When higher triglycerides, such as tripalmitin, served as substrates, the optimum was on the acid side. A recent comprehensive investigation of plant lipases by Bamann and Ullmann (1942) showed that the optimum hydrogen-ion concentrations of lipases of vegetative organs, and of most ungerminated seeds, lie between *p*H 8.5–10.5. These investigators, employing tributyrin as the substrate and the stalagmometric technique, found that, of all the plants investigated, only the lipase of the mature ungerminated *Ricinus* showed an optimum on the acid side of neutrality, namely, at *p*H 4.7. The optima of the immature seeds and of the vegetative organs of the castor bean plant are between *p*H 8.5 and 10.5. Changes in the optima were observed at different physiological states as follows:

Sample	*p*H optimum
Immature seed	8.5
Mature, ungerminated seed	4.7
Germinated seed	6.8

This behavior may also be characteristic of the cereal grains and would be worthy of investigation.

Obviously, optimum hydrogen-ion concentrations will vary depending upon the type of buffer and the substrate employed. Methods have been described by which a continuous electrometric titration in a buffer-free medium has been successfully used for determining the optimum; these have been applied in studying animal lipases and should prove very useful in research on the cereal grains.

Temperature. Temperature optima have not been determined for the plant lipases, but most determinations have been made between 30 to

38°C, except in the stalagmometric procedure where 20°C is the prescribed temperature.

Time. The time used varies with the method employed and may range from several minutes to 24 hours.

OCCURRENCE OF THE LIPOLYTIC ENZYMES IN BAKING MATERIALS

Sound Wheat. It has been known for years that a fat-splitting enzyme occurs in wheat, but it is usually mentioned quite casually with no details about its measurement. Data presented by Iono (1931) confirm earlier observations that esterases are present in wheat seeds and that their activity increases during germination. In a study of the effect of enzymes on breadmaking, Borasio (1932) measured the lipase activity (reported as grams of butyric acid liberated) of six varieties of wheat, and concluded that this enzyme was localized in the outer layers of the kernel. Kiezel and Gordienko (1937) found that the lipase activity of wheat increased as the moisture content of the grain was raised to 14.5 or 15.0 per cent. Conducting their experiments on a hard and a soft wheat, Kretovitch, Sokolova, and Uschakova (1940) observed that fat-splitting activity and moisture content are directly correlated. These investigators actually determined the acidity of the fat (extracted from wheat by ether) by titration with 0.01 *N* alkali and phenolphthalein; their results were expressed as the ml of 0.01 *N* alkali per g of dry wheat. Part of these data are given in Table I. Secchi

TABLE I

ACIDITY OF FAT EXTRACTED FROM WHEAT BY ETHER (Kretovitch, Sokolova, and Uschakova, 1940)

Moisture content		Acidity[1] of ether extract	
Hard wheat	Soft wheat	Hard wheat	Soft wheat
%	%	%	%
19.1	19.3	0.61	0.63
13.7	14.2	0.37	0.42
12.1	12.5	0.29	0.39
9.5	9.9	0.26	0.35

[1] Ml of 0.01 *N* alkali per g of dry wheat.

(1942), employing the method of Willstätter and Waldschmidt-Leitz with a temperature of 32°C and a *p*H of 4.6, found greater lipolytic activity in hard than soft wheat and observed that the activity disappeared when the material was warmed for 20 minutes at 60°C.

In a preliminary investigation of the lipolytic enzymes of wheat,

Sullivan and Howe (1933) found that these enzymes act on the glycerides of the fatty acids to a greater extent than on the corresponding esters. Glycerides of the lower fatty acids, particularly triacetin and triproprionin, were hydrolyzed to the greatest extent, triacetin giving notably higher results than any other substrate tried. Ethyl acetate gave a higher titration value than ethyl butyrate, which showed very little hydrolysis, but neither of these esters was acted on to the same extent as triacetin and triproprionin. The larger titration values given by triacetin and ethyl acetate, by comparison with those of the higher members of the series, are undoubtedly due to the greater miscibility of these lower members in the medium.

Table II illustrates some of the results obtained with ground wheat as the enzyme source and triacetin as the substrate. The ground wheat was extracted with ether, toluene, glycerol, water, trypsin, and diastase in an effort to obtain a greater concentration of the lipase. None of these attempts succeeded in giving any significant increase in titration values as compared with the original ground wheat.

TABLE II

GROUND WHEAT EXTRACTED BY VARIOUS METHODS IN AN EFFORT TO INCREASE THE CONCENTRATION OF THE LIPASE WHICH ACTS ON TRIACETIN
(From Sullivan and Howe, 1933)

Results expressed in ml of $N/10$ sodium hydroxide

Product and treatment	Hydrogen-ion concentration, pH					
	5.8[1]	7.7	7.4	6.3	5.8	5.2
Resting wheat						
Not extracted	22.1	27.4	25.9	20.1	17.9	14.4
Exhaustive ether extraction	23.1	29.0	27.0	21.8	17.6	12.8
Exhaustive toluene extraction	22.6	28.0	25.9	21.3	17.5	14.6
Preliminary digestion with trypsin						
Residue not extracted	2.5	2.9	3.1	4.5	3.2	2.5
Residue extracted with ether	3.1	4.2	3.1	4.0	3.3	2.0
Filtrate from tryptic digestion	19.5	24.1	23.5	—	—	—
Preliminary digestion with diastase						
Residue extracted with ether	3.1	3.0	4.0	4.0	3.7	3.2
Filtrate	13.5	19.1	19.0	—	—	—
Glycerol extraction	13.6	19.7	19.7	—	—	—
Water extraction						
Residue	7.1	10.1	9.2	7.8	6.3	3.8
Filtrate	8.4	17.9	16.9	12.6	8.9	6.8
Germinated wheat	22.5	29.0	27.5	22.0	18.4	16.5

[1] No buffers.

When compared with many other higher plants, and particularly with the oil-bearing seeds such as the castor bean, wheat may be considered a poor source of lipolytic enzymes. Bamann and Ullmann (1942) state that there is no parallelism between the fat content of a seed and its lipolytic activity. According to these investigators, the lipase content of plants varies

with the state of maturity and the age of the seed. Lipase activity decreases with increasing age. No figures were given for wheat.

Germinated Wheat. The lipolytic activity of wheat and of other higher plants increases on germination and is usually more pronounced at

TABLE III

EFFECT OF GERMINATION ON THE HYDROLYSIS OF THE GLYCERIDES OF THE HIGHER FATTY ACIDS (From Sullivan and Howe, 1933)

*p*H	Treatment	Trimyristin	Tripalmitin	Tristearin	Wheat fat (ether-extracted)
	Unextracted wheat				
5.2	Germinated (three days)	3.2	2.6	1.8	2.2
5.2	Resting	1.5	0.7	0.4	0.7
	Ether-extracted wheat				
5.2	Germinated previous to extraction	—	—	—	2.2
5.2	Resting	—	—	—	1.1

higher moisture levels. According to Sullivan and Howe (1933), the germination of wheat increases the activity of its lipase when the higher triglycerides serve as the substrates, but makes very little difference when the glycerides of the lower fatty acids are employed. Table III gives their results with the higher glycerides and illustrates the increased activity of the enzyme on germination.

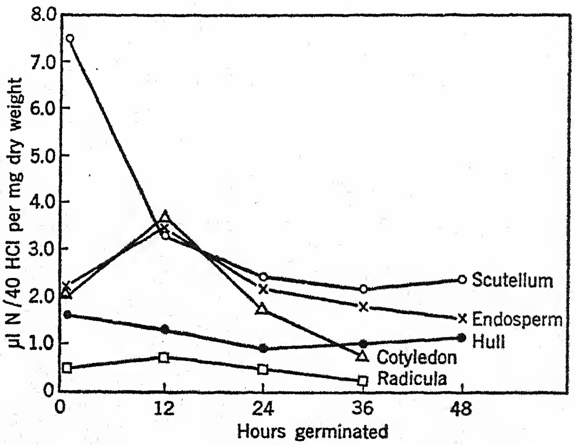

Fig. 1.—Lipase activity in different parts of wheat seeds during germination (Pett, 1935).

A valuable contribution by Pett (1935) described the changes that occur in the esterase activity of various parts of the wheat kernel when it is germinated. He dissected Manitoba wheat, both dormant and germinated,

under a tripod lens and obtained five easily separated fractions: the hull (including epidermal, aleurone, and parenchymatous cells); the endosperm; the scutellum; and the embryo cut arbitrarily into two sections—the cotyledon and radicles. Separate analyses of histologically defined portions are ideal for determining enzyme distribution.

The results are illustrated graphically in Figure 1. Using methyl butyrate as the substrate, Pett found that the highest esterase content was associated with the scutellum, and that there was a sudden drop of activity in this fraction on germination. The endosperm showed a high esterase

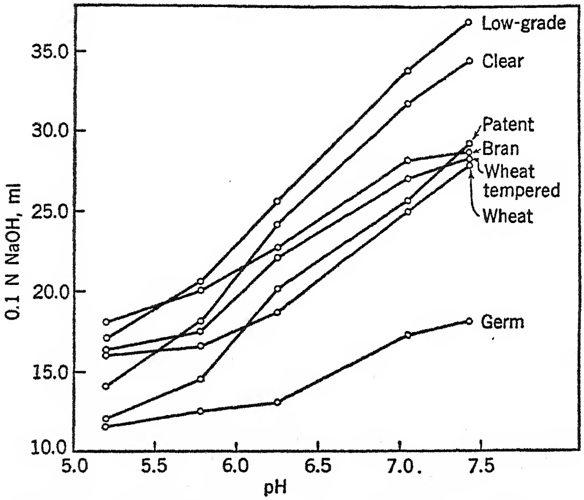

Fig. 2.—The relations between lipase activity and hydrogen-ion concentration, with KH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$ as buffers, for wheat and wheat products (Sullivan and Howe, 1933).

content, but one which was much lower than that of the scutellum. The esterase activity of the endosperm was found to increase in the first twelve hours of germination and subsequently to decrease gradually. The greater part of the esterase activity of the embryo was in the cotyledon; this increased during the first twelve hours of germination and then decreased sharply. The hull or bran contained a significant amount of esterase, and no change in its activity occurred on germination.

Products of Milling. It has been generally assumed that wheat germ, which has the highest fat content of all the fractions separated by milling, possesses the highest lipolytic activity. However, in comparing the usual milling separations of wheat, Sullivan and Howe (1933) found that other fractions hydrolyzed the lower triglycerides to a

much greater degree. Figures 2 and 3 show that clear and low-grade flours possess greater activity than patent flours, germ, or bran. The lipases are not concentrated in the bran layers but seem to be present in largest amount in the scutellum and the aleurone layer. The work of Sullivan and Howe and of Pett, on the distribution of these enzymes, constitutes the only studies made on this subject.

Other Materials. Yeast contains lipase, and its optimum hydrogen-ion concentration with olive oil as the substrate, is given as *p*H 6.6 to 6.8; its optimum temperature is reported as 30°C (Gorbach and Günter, 1932). The enzyme is injured by cell poisons such as chloroform, ethyl acetate, and ether. It is worth mentioning that another group of esterases, the

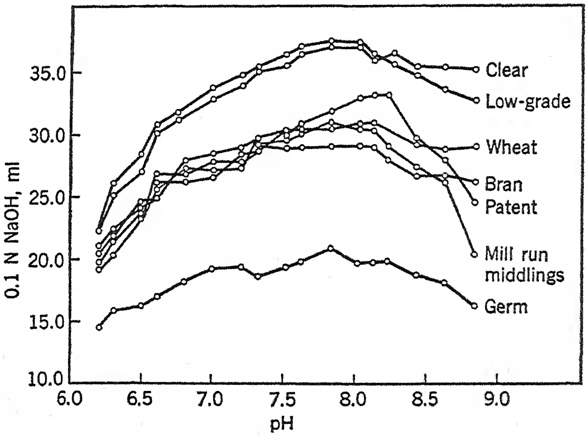

Fig. 3.—The relations between lipase activity and hydrogen-ion concentration, with 0.1 *M* KH_2PO_4 and 0.5 *M* $Na_2B_4O_7 \cdot 10\ H_2O$ as buffers, for wheat and wheat products (Sullivan and Howe, 1933).

phosphatases, are present in yeast and are capable of hydrolyzing phenyl phosphate (Rae and Eastcott, 1940) and α- and β-glycerophosphate (Kertesz, 1930). Cocarboxylase is also split by a pyrophosphatase from yeast (Westenbrink, Van Dorp, Gruber, and Veldman, 1940). Milk and some malt extracts also contain small amounts of lipase.

THE LIPIDES IN BAKING MATERIALS

Wheat and Its Products. Since the lipolytic enzymes must act, in storage and in breadmaking, on the lipides of the products containing them, rather than on the synthetic substrates usually used for their measurement, some knowledge of the distribution and constitution of the lipides of wheat

and its products is essential to an understanding of the activity of their lipolytic enzymes.

The distribution of the lipides in various separations milled from hard spring wheat is as follows (Sullivan, 1940):

Sample	Per cent lipides (alcohol-ether extract)
Wheat	3.0
Short patent	1.3
Straight	1.6
Clear	2.7
Low-grade	3.2
Bran	6.5
Shorts	7.5
Germ (good commercial separation)	15.5

These figures are only approximate and will naturally vary with the type of wheat, the percentage extraction, and the method of milling.

The lipides of wheat germ, which have been more extensively investigated than those of any other wheat product (Jamieson and Baughman, 1932; Channon and Foster, 1934; Sullivan and Bailey, 1936), are composed of approximately 4 per cent of unsaponifiable material, some lecithin and cephalin (4 to 10 per cent), and the balance of mixed triglycerides. On hydrolysis, the triglycerides give the following percentages of fatty acids: palmitic, 11.8; stearic, 3.0; lignoceric, 1.2; oleic, 28.1; linoleic, 52.3; and linolenic, 3.6 per cent. Approximately 16 per cent of saturated fatty acids are present. As might be expected from these figures, no fully saturated glycerides are present—the fat is composed largely of mixed monosaturated-diunsaturated glycerides and triunsaturated glycerides (Sullivan and Bailey, 1936).

The lipides of wheat flour contain more volatile acids than those of wheat germ. As is the case with the lipides of wheat germ, the bulk of the saturated fraction, which amounts to roughly 15 per cent, is palmitic acid. The unsaturated acids, oleic, linoleic and linolenic, are the same as those present in wheat germ, with linoleic acid predominating (Sullivan and Howe, 1938). Other fractions of flour and feed do not show wide variations in the distribution of the fatty acids present in their glycerides.

Barton-Wright (1938a) has investigated the distribution of fats in the wheat kernel by a staining technique using Sudan IV. The aleurone layer and the bran showed the presence of fat globules but no fat could be seen in the endosperm. The epithelial layer next to the endosperm and the scutellum were heavily charged with fat. The fat in the embryo, scutellum, and epithelial cells was observed to be evenly distributed throughout the cell protoplasm, and globules of fats were never discovered; these globules were apparently largely restricted to the aleurone layer.

Shortening. The glycerides of the shortenings commonly used in

baking (hydrogenated vegetable oils, lard, and butter) are composed of the straight-chain, even-numbered, fatty acids from butyric to lignoceric with the major portion made up of fatty acids with sixteen to eighteen carbon atoms. These may vary from the saturated acids to those containing four double bonds. In most cases oleic acid is the predominant unsaturated acid. The possible number of mixed glycerides naturally present in the various vegetable oils used in making shortening is legion. In addition, there are the triglycerides resulting from hydrogenation and mono- and diglycerides added artificially to special types of shortenings used mainly in cake production.

Other Products. The lipides of yeast, milk, malt, and other baking ingredients, except shortening, are usually present in such small amounts that they are probably negligible from the standpoint of their effect on fermentation.

ROLE OF LIPOLYTIC ENZYMES

Storage of Grain. The deterioration of grain in storage is usually accompanied by an increase in the acid number of the fat. Respiration and heating, as well as enzyme activity, are more pronounced at the higher moisture levels. Zeleny and Coleman (1938, 1939) have shown that the fat acidity of grain, and particularly of corn, is a more reliable measure of the degree of soundness than any other chemical or physical test thus far employed. Fat acidity is considered by Zeleny (1940) merely as a useful index of the more obscure and complex changes occurring during the respiration and heating of grain. It is possible for grain that has been stored for a long time under adverse conditions to become unfit for human consumption even when there has been no heating. The role of the lipases (which may be largely responsible for the increase in the acidity of the fat) has not been studied in connection with storage problems nor has there been any investigation of the relationship of lipolytic activity to respiration in stored grain. This subject would constitute an interesting research problem.

Storage of Flour and Other Milled Products. The hydrolysis of flour fat by its esterases is much more rapid at high moisture levels and elevated storage temperatures than at normal moisture and temperatures.

It has been shown by Sullivan and Near (1933) that the alcohol-ether extract of wheat and its milled products drops considerably in samples stored at or near their original moisture of 13 to 14 per cent. Hartmann (1930) has suggested that the age of a flour can be estimated by the uniform increase in acid value up to at least fifteen months. His results were not substantiated by Barton-Wright (1938), who found that in normal

flour the acid value increases on storage to a maximum and then falls off. In moistened samples of flour, the petroleum ether extract was found to decrease rapidly during storage. The bacterial count decreased, but the fungal count increased greatly with storage of high-moisture samples. Since the acid value of the oil increases during storage, Barton-Wright considered that the lipase of the flour, and not of the bacteria or fungi, was responsible; a chloroform-treated sample proved to be completely sterile and still showed an increase in acid number.

If flour is stored in a cool, dry place, its baking quality is usually maintained or actually improved over a period of several months. Storage for longer periods, especially in a warm, humid climate, causes flour to deteriorate and become unsatisfactory for breadmaking. Flour damaged in this way exhibits a "short" gluten, and the dough made from such flour lacks extensibility and tears easily. The volume, flavor, and taste of the finished bread are inferior.

The mechanism of these changes is interesting to follow. It was first observed by Kozmin (1934, 1934a, and 1935), then by Sullivan, Near, and Foley (1936), and finally by Barton-Wright (1938), that unsaturated fatty acids—such as oleic, linoleic, and linolenic—produce short, brittle glutens. Sullivan *et al.* (1936), and later Barton-Wright (1938), found that, in spite of their deleterious effect on gluten, small amounts of the unsaturated acids do not injure the breadmaking characteristics to the extent that might be expected from their harmful action on gluten. It was demonstrated by Sullivan and co-workers (1936) that a pronounced damaging action on the breadmaking characteristics occurred only when these unsaturated acids became oxidized, either by air or by the oxidizing enzymes present in the flour. The oxidized unsaturated fatty acids produce a dough which is "dead" and a bread with poor volume, flavor, and taste. Since the glycerides of flour are composed largely of unsaturated fatty acids (mainly linoleic), the production of unsaturated acids is sufficient to harm the flour even though its total fat content is not much more than 1 to 2 per cent.

Natural or manufactured products containing fat may develop either the hydrolytic or oxidative type of rancidity. Frequently both are present, but one kind usually predominates. Not enough work has been done on the cereal grains to make authoritative statements, but it appears from data thus far available that lipolysis and oxidative rancidity may occur together in wheat products. Rancidity in wheat products, however, is usually due to oxidation. On storage, the acid numbers of the fats of flour and feeds always increase and probably a large proportion of this increase is due to lipolytic action. It has never been determined how much, if any, of the change in the acid numbers of the fats extracted from wheat

and its milled products is due to nonenzymatic hydrolysis. Hence, it is hardly valid to assume, as some investigators have done, that the increase in acid numbers of the fat constitutes an accurate measurement of lipolytic activity.

It is generally accepted that a bleached flour tends to go out of condition more rapidly than unbleached flour of the same grade and from the same wheat mix, and that soft wheat flours age more rapidly than hard wheat flours; also, lower-grade flours are more likely to go out of condition than short patents. Under normal conditions, flour keeps very well. Except in rare crop years or in certain isolated instances in which storage conditions have been adverse or too prolonged, loss of flour due to poor baking characteristics resulting from lipolysis or oxidative rancidity has not constituted a serious economic problem.

The acidity of flour increases with age and with increasing temperature during storage, and acidity determinations have been proposed as a measure of the soundness of flour. Balland (1903) presented evidence to show that the increased acidity resulting from aging was due mainly to fatty acids.

In 1924, the Greek Ministry of Health imposed certain maximum limits of acidity on flour imported to Greece; the maximum allowable acidity was 0.15 per cent for second quality flours (clears and low grades). This figure was based on results obtained by titration with alkali of an 85 per cent ethanol extract of flour using curcuma as an indicator, and acidity was reported as sulfuric acid. Considerable difficulty resulted from this regulation from 1927 to 1929 when United States mills exported clear and low-grade flours to Greece and many of these flours were found to exceed the maximum limits for acidity. Since the acidity of flours increases with increasing temperature, more trouble was experienced during hot weather. An acidity of 0.15 per cent has been shown to be too low to serve as a measure of soundness, since many clear and low-grade flours exceed this figure and still possess entirely satisfactory breadmaking characteristics (Fifield and Bailey, 1929). Some methods for the determination of acidity in which water extracts are employed measure not only the fatty acids developed on lipolysis, but other acidic constituents such as amino acids and phosphoric acid resulting from hydrolysis or enzyme activity. The various methods of acidity determinations have been discussed in detail by Bailey (1944). Although several foreign countries specify a maximum acidity for various grades of imported flours, acidity determinations are not used in routine practice in the United States or Canada as a measure of soundness.

During Fermentation and Baking. In spite of the fact that the conditions of fermentation are conducive to the activity of the fat-splitting

enzymes, these do not appear to have much effect on the dough. The temperature of baking probably inactivates these enzymes, and hence they are of no importance in the keeping quality of the baked products. Rancidity in baked products is almost always due to oxidation.

PHOSPHATASES

Phosphatases or phosphoesterases are enzymes hydrolyzing various esters of phosphorus oxy acids. These numerous enzymes have a most important function in the metabolism and synthesis of carbohydrates. Although certain phosphatases have been known to be present in cereals, little has appeared in the literature until recently concerning these enzymes in wheat.

Berliner and Rüter (1928) found that wheat flour extracts contained a phosphatase capable of dephosphorylating soybean phosphatide. Herd (1931) employed conductivity measurements of flour extracts acting on soybean suspensions, and also on flour, to measure the increase in phosphoric acid resulting from hydrolysis. The decrease in phosphatase activity on heating flour, observed by Berliner and Rüter, was not confirmed by Herd for flour heated for 10 hr at 82°C or less. The heat treatment used by Berliner and Rüter was more severe than that employed by Herd, and in any case, the method used by both is too general to throw any light on the specific phosphatases involved.

Phosphomonoesterase, Phosphodiesterase. A phosphomonoesterase and a phosphodiesterase were found in rice bran by Uzawa (1932). The monoesterase hydrolyzed several phosphate substrates including β-glycerophosphate and phenyl phosphate and had an optimum hydrogen-ion concentration of about pH 5.5. The distribution of phosphomonoesterase in wheat before and during germination was measured by Ignatieff and Wasteneys (1936). The leaves were found to have a higher concentration of phosphatase than any other part of the plant. Sarma and Giri (1942) found that the phosphomonoesterase of rice and ragi may exist in both "free" and "bound" conditions, the "free" form being water soluble while the "bound" form is not extracted by water. The significant increase in the total phosphatase content of rice during germination was attributed to freeing of the "bound" phosphatase.

The phosphatase activity in leaf blades of young sprouts of wheat was investigated by Sisakyan and Kobyakova (1940). The synthesizing activity of the phosphatases was decreased and the hydrolyzing activity increased when 30–40 per cent of water was lost from the leaves. When 40–50 per cent of the water was lost, both the synthesizing and hydrolyzing activity of the phosphatase was reported to increase.

An excellent paper by Booth (1944) has clarified and extended the preliminary observations on the phosphatases of wheat. Booth described two methods for the measurement of free phosphomonoesterase activity, both of which are modifications of methods previously developed for use with blood plasma and animal tissues. An optimum of pH 5.15 was established for the "free" phosphomonoesterase. The activity disappeared between pH 3.0 to 4.0 and also between pH 6.0 to 7.0. This phosphatase in solution had a critical inactivation temperature of 53° and the thermal destruction was found to be irreversible. Neither magnesium sulfate nor hydrogen cyanide activated the enzyme. Sodium fluoride inhibited the activity greatly, but 10^{-2} to 10^{-6} M iodoacetic acid gave no inhibition. Evidence presented by Booth indicates there are at least two phosphomonoesterases in wheat, one of which hydrolyzes α-glycerophosphate more rapidly than β-glycerophosphate. The total water-soluble phosphomonoesterase activity of wheat is also greater when α- rather than β-glycerophosphate is used as the substrate. Booth's results also indicate the presence in wheat of a hexosediphosphatase, pyrophosphatase, and a phosphodiesterase.

Pyrophosphatase. Pyrophosphatase, a different enzyme from the phosphomonoesterase previously described, was found by Giri (1941) to occur in rice, barley, and maize.

Obermeyer, Fulmer, and Young (1944) observed that no enzyme addition was required to convert cocarboxylase (the pyrophosphoric ester of thiamine) to free thiamine in testing a flour to which synthetic cocarboxylase had been added. This indicated that flour contained an enzyme capable of hydrolyzing cocarboxylase to thiamine and also that the failure to find cocarboxylase in many plant materials might be caused by phosphatase activity when the materials were extracted during preparation for assay. It was demonstrated by these investigators, in support of previous workers, that the natural thiamine of wheat does not occur as cocarboxylase; hence an enzyme digestion is not required for the estimation of natural thiamine in wheat flour.

Adenosinetriphosphatase. The presence in wheat of a most important enzyme, adenosinetriphosphatase, was demonstrated recently by Glick and Fischer (1945) using Gomori's technique for the histochemical localization of phosphatases. This method depends on the precipitation *in situ* of the phosphate, liberated enzymatically, by calcium in alkaline media and lead in acid media. The insoluble phosphate is converted to the more easily observed black lead sulfide. A hanging drop technique was developed that required only a small drop of substrate for each tissue section. Both glycerophosphatase and thiamine pyrophosphatase were demonstrated in wheat by this method. Previously, Hilbe and Marron (1940)

had attempted to localize glycerophosphatase in wheat sections by the Gomori technique.

Without question, other phosphatases as well as phosphorylating mechanisms will be found in wheat and its products. These enzymes, and the oxidation-reduction reactions in which they participate, undoubtedly will be found to be of great importance in the carbohydrate metabolism of germinating seeds and plants, just as they have already been found to play vital roles in the carbohydrate metabolism of animals. Fermentation depends on many of these same reactions, and our understanding of the behavior of bread doughs will be illuminated by further research on the many phosphatases and coenzymes of flour and yeast and of the oxidation-reduction reactions occurring during fermentation.

Phytase. This enzyme is an esterase that hydrolyzes phytic acid or phytin to inositol and phosphoric acid or its salts. It was first discovered in rice (Suzuki, Yoshimura, and Takaishi, 1907) and is present in barley (Vorbrodt, 1910; Adler, 1916) and wheat (Collatz and Bailey, 1921). Phytase is more abundant in the pericarp than in other parts of the grain. It is usually prepared by water extraction of finely ground bran from wheat or rice at a temperature of 2 to 3°C, with subsequent precipitation of the enzyme by filtering the water solution into ethanol. Further purification may be effected by drying the preparation, dissolving it in water, and reprecipitating it with ethanol. The enzyme preparation from barley malt is reported to have an optimum *p*H of 5.4 to 5.5 (Adler, 1916). Pringle and Moran (1942) cite *p*H 5.2 to 5.3 as optimum for the phytase of wheat products.

The amount of phosphoric acid liberated under prescribed conditions may be used as an index of the activity of phytase. Since phosphoric acid and its salts are end-products of the hydrolysis of phytin and are ionized to a greater extent in water solution than the original phytin, the specific conductivity of a solution of phytin and phytase was proposed by Collatz and Bailey (1921) as a convenient measure of the activity of the enzyme. According to these investigators, a phytase preparation from wheat bran caused a more complete hydrolysis of phytin at a temperature of about 55°C than at any other temperature, although hydrolysis was more rapid at 60°C during the first 15 minutes. No buffer was used in these experiments.

It is assumed that phytase uses as a substrate either phytic acid (the phosphoric acid ester of inositol) or phytin. Phytin is reported to exist in wheat and in many other grains as the calcium-magnesium salt of phytic acid. Apparently, phytase is effective in flour doughs, yet, according to Pringle and Moran (1942), phytase cannot break down phytates when these salts are in an insoluble form. It is not clear if both phytic

acid and its salts are equally effective as substrates; the probability is that they are not. Moreover, when phytin is the substrate, the calcium and magnesium ions would be expected to have some influence on the enzyme reaction. Phytase is not well characterized and further research is badly needed. So far as the writer is aware, there are no data on the effect of changing the concentration of enzyme and substrate, or on the influence of buffers and other allied factors that might lead to a better understanding of the activity of this enzyme. Furthermore, some fraction of the observed cleavage of phytin may be due to nonenzymatic hydrolysis.

Bailey and Collatz (1921) observed that the specific conductivity of flour extracts parallels the ash content and can be used as an index of flour grade. Strict adherence to uniform conditions of preparation of the extract, and of time and temperature, are essential if comparisons of flours are made. From the similarity of the response of flour extracts and of phytin–phytase preparations to temperature changes, it was assumed that the conductivity of water extracts of flour was due mainly to inorganic salts of phosphoric acid resulting from hydrolysis of phytin by phytase. Bailey and Peterson (1921) observed that buffer action parallels the specific conductivity of flour–water extracts, and they believed that the phosphates produced by the action of phytase were the principal buffers of the extracts. Johnson (1925) found that the activity of phytase is not an important factor in accounting for the changes in acidity that occur in fermentation. Acid phosphate salts resulting from phytase activity had a very slight effect on the final acidity of flour–water suspensions and of cracker sponges fermented with yeast.

It has been suggested by Hay (1942) that the water-insoluble calcium and magnesium salt of phytic acid, present in the pericarp of the wheat kernel, may insure the phosphorus from loss by diffusion when the grain is exposed to rain, but that the phosphorus is liberated in available inorganic form by the action of phytase when the germinating plant requires it.

Recently, phytic acid has been the object of increased attention because it can immobilize calcium and iron and make both these elements less available for nutrition. Several investigators, such as Andrews and Bailey (1932), Pringle and Moran (1942), and Hay (1942), have shown that between 85 and 100 per cent of the total phosphorus of wheat bran is present as phytate. Flour, on the other hand, has only 8 to 9 per cent of the phosphorus content of bran, and, of this amount, only 24 to 31 per cent is present as phytic acid phosphorus (Hay, 1942; Pringle and Moran, 1942).

McCance and Widdowson (1942) have demonstrated that the availability, and hence the optimum intake of calcium in humans, is dependent on the amount of phytic acid in the diet. If an excess of phytic acid is

present, as might occur when relatively large amounts of bran or long-extraction flour are consumed, more calcium must be provided. The poor absorption of calcium and magnesium from whole wheat and from long-extraction flours is due entirely to phytic acid according to McCance and Widdowson (1942a). They demonstrated this both by removing phytic acid enzymatically from long-extraction flour, and by adding sodium phytate to white flour. On the basis of these experiments, the Medical Research Council of England recommended that the 85 per cent National Wheatmeal be fortified with calcium.

Utilizing "human balance" experiments and serum–iron curves, McCance, Edgecombe, and Widdowson (1943) noted a definite interference in iron absorption in the presence of sodium phytate and conclude that the phytates of whole cereals should have a similar effect and inhibit the absorption of iron.

Since phytic acid interferes with the utilization of both calcium and iron, it is interesting to note the observation of Widdowson (1941), who stated that phytase is active in commercial flours and that 85 per cent of the phytic acid contained in white flour is destroyed by this enzyme during the processes of breadmaking. In view of its important effect on the nutritional value of cereals, phytase is deserving of more careful and extensive research.

POSSIBILITIES FOR FUTURE RESEARCH

Before the functions of the esterases of the cereal grains can be fully understood, it is essential to secure certain fundamental data concerning the properties of the enzymes themselves. The optimal hydrogen-ion concentration for the action of the lipolytic enzymes should be determined for a series of both glycerides and esters in systems free from other factors that might influence the activities. The specific effect of various salts used as buffers and the effect of other compounds such as bleaching agents and improvers should be studied to determine their possible influence on lipolytic activity. Much more basic data are needed concerning the phosphatases before their role in cereal products can be properly evaluated. In addition to the establishment of hydrogen-ion optima for the lipolytic enzymes and the numerous phosphatases, it is also necessary to determine temperature optima, the effects of varying enzyme and substrate concentrations, and specificity. When such basic experimental data are secured, the effect of the esterases on the storage of cereal grains and their milled products, and on other problems affecting the milling and baking industry, can be better evaluated.

BIBLIOGRAPHY

ADLER, L. (1916). Gewinnung von Phytase aus Malz. *Biochem. Z.* 75:319–338.

ANDREWS, J. S., and BAILEY, C. H. (1932). Distribution of organic phosphorus in wheat. *Ind. Eng. Chem.* 24:80–82.

BAILEY, C. H. (1944). *Constituents of Wheat and Wheat Products.* Reinhold, New York.

—— and COLLATZ, F. A. (1921). Studies on wheat flour grades. I—Electrical conductivity of water extracts. *J. Ind. Eng. Chem.* 13:319–321.

—— and PETERSON, A. C. (1921). Studies of wheat flour grades. II—Buffer action of water extracts. *J. Ind. Eng. Chem:* 13:916–918.

BALLAND, A. (1903). Sur les matières graisses et l'acidité des farines. *Compt. rend.* 137:724–725.

BAMANN, E., and ULLMANN, E. (1942). Untersuchungen über die Lipase höherer Pflanzen. *Biochem. Z.* 312:9–40.

BARTON-WRIGHT, E. C. (1938). Studies on the storage of wheaten flour. III. Changes in the flora and the fats and the influence of these changes on gluten character. *Cereal Chem.* 15:521–541.

—— (1938a). Observations of the nature of the lipides of wheat flour, germ and bran *Cereal Chem.* 15:723–738.

BERLINER, E., and RÜTER, R. (1928). Die Wirkung der Hitze auf Mehl. *Z. ges. Mühlenw.* 4:209–216.

BOOTH, R. G. (1944). Cereal phosphatases. *Biochem. J.* 38:355–362.

BORASIO, L. (1932). Enzymes and panification. *Giorn. risicoltura* 22:137–149. (*Chem. Abstracts* 27:346.)

CHANNON, H. J., and FOSTER, C. A. M. (1934). The phosphatides of the wheat germ. *Biochem. J.* 28:853–864.

COLLATZ, F. A., and BAILEY, C. H. (1921). The activity of phytase as determined by the specific conductivity of phytin-phytase solutions. *J. Ind. Eng. Chem.* 13:317–318.

DAKIN, H. D. (1904). The hydrolysis of optically inactive esters by means of enzymes. Part I. The action of lipase upon esters of mandelic acid. The resolution of inactive mandelic acid. *J. Physiol.* 30:253–263.

—— (1905). The fractional hydrolysis of optically inactive esters by lipase. Part II. *J. Physiol.* 32:199–206.

FIFIELD, C. C., and BAILEY, C. H. (1929). The march of acidity in stored flours. *Cereal Chem.* 6:530–540.

GIRI, K. V. (1941). Plant phosphatases. II. Glycero- and pyrophosphatase and phytase activities of plant tissues. *Ann. Biochem. Exptl. Med.* 1:297–306.

GLICK, D. (1934). Beiträge zur enzymatischen Histochemie. VIII. Eine Mikromethode zur Bestimmung der Aktivät lipolytischer Enzyme. *Z. physiol. Chem.* 223:252–256.

—— and FISCHER, E. (1945). The histochemical localization of adenosinetriphosphatase in plant and animal tissues. *Science* 102:429–430.

GORBACH, G., and GÜNTER, H. (1932). Über Hefelipase. *Monatsh.* 61:47–60.

HARTMANN, W. (1930). Über den Nachweis des Alters bei Mehlen. *Z. Untersuch. Lebensm.* 59:364–379.

HAY, J. G. (1942). The distribution of phytic acid in wheat and a preliminary study of some of the calcium salts of this acid. *Cereal Chem.* 19:326–333.

HERD, C. W. (1931). A study of some methods of examining flour, with special reference to the effects of heat. II. Effects of heat on flour enzymes. *Cereal Chem.* 8:145–161.

HILBE, J. J., and MARRON, T. V. (1940). Differences in the phosphatase systems of plant and animal tissues by a microtechnic. *Proc. Iowa Acad. Sci.* 47:235–237.

IGNATIEFF, V., and WASTENEYS, H. (1936). Phosphatase distribution in some higher plants. *Biochem. J.* 30:1171–1182.

IONO, Y. (1931). Vergleichende Untersuchungen über den Fermentgehalt der ruhenden und keimenden Pflanzensamen. *Acta Schol. Med. Univ. Imp. Kioto* 13: 211–238.

JAMIESON, G. S., and BAUGHMAN, W. F. (1932). Wheat germ oil. *Oil & Soap* 9: 136–138.

JOHNSON, A. H. (1925). Identification and estimation of the organic acids produced during bread dough and cracker dough fermentation. *Cereal Chem.* 2:345–364.

KERTESZ, Z. I. (1930). The acidity optimum of yeast hexosediphosphatase. *J. Am. Chem. Soc.* 52:4117–4119.

KIEZEL, A., and GORDIENKO, K. (1937). Über die Tätigkeit der Fermente in Weizenkorn von verschiedenem Wassergehalt während der Lagerung. *Bull. soc. naturilistes Moscou* 46:339–349.

KOZMIN, N. P. (1934). Reifung der Weizenmehle und ihre biochemischen Grundlagen. *Mühlenlab.* 4:18–32.

—— (1934a). Kleberqualität und einige Faktoren, die diese beeinflussen. *Mühlenlab.* 4:109–116.

—— (1935). The aging of wheat flour and the nature of this process. *Cereal Chem.* 12: 165–171.

KRETOVITCH, V. L., SOKOLOVA, A. I., and USCHAKOVA, E. N. (1940). Über die stabile Feuchtigkeit des Kornes und ihren Einfluss auf die Arbeit der Lipase. *Compt. rend. acad. sci. U.R.S.S.* 27:701–704.

LINDERSTRØM-LANG, K., and HOLTER, H. (1934). Enzymatische Histochemie. *Ergeb. Enzymforsch.* 3:309–334.

LONGENECKER, H. E., and HALEY, D. E. (1935). *Ricinus* lipase, its nature and specificity. *J. Am. Chem. Soc.* 57:2019–2021.

McCANCE, R. A., EDGECOMBE, C. N., and WIDDOWSON, E. M. (1943). Phytic acid and iron absorption. *Lancet* 2:126–128.

—— and WIDDOWSON, E. M. (1942). Mineral metabolism of healthy adults on white and brown bread dietaries. *J. Physiol.* 101:44–85.

—— and —— (1942a). Mineral metabolism on dephytinized bread. *J. Physiol.* 101:304–313.

OBERMEYER, H. G., FULMER, W. C., and YOUNG, J. M. (1944). Cocarboxylase hydrolysis by a wheat phosphatase. *J. Biol. Chem.* 154:557–559.

OLCOTT, H. S., and FONTAINE, T. D. (1941). Composition of cottonseeds. IV. Lipase of germinated seed. *J. Am. Chem. Soc.* 63:825–827.

PETT, L. (1935). Studies on the distribution of enzymes in dormant and germinating wheat seeds. II. Lipase. *Biochem. J.* 29:1902–1904.

PRINGLE, W. J. S., and MORAN, T. (1942). Phytic acid and its destruction in baking. *J. Soc. Chem. Ind.* 61:108–110.

RAE, J. J., and EASTCOTT, E. V. (1940) The estimation of phosphatase in yeast. *J. Biol. Chem.* 136:443–447.

SARMA, M. L., and GIRI, K. V. (1942). Plant phosphatases. IV. Occurrence of "free" and "bound" phosphatases in seeds. *Ann. Biochem. Exptl. Med.* 2: 17–24.

SECCHI, G. (1942). Attivita lipolitica die derivati di cereali e de leguminose. *Ann. chim. applicata.* 32:115–120. (*Chem. Abstracts* 36:7153.)

SISAKYAN, N. M., and KOBYAKOVA, A. (1940). The prevailing direction of enzyme action as an index of drought resistance in cultivated plants. IV. The effect of withering upon the trend of the process of esterification and hydrolysis of phosphoric esters in plants. *Biokhimiya* 5:225–233.

SULLIVAN, B. (1940). The function of the lipids in milling and baking. *Cereal Chem.* 17:661–668.

—— and BAILEY, C. H. (1936). The lipids of the wheat embryo. I. The fatty acids. *J. Am. Chem. Soc.* 58:383–390.

—— and HOWE, M. A. (1933). Lipases of wheat. I. *J. Am. Chem. Soc.* 55:320–324.

—— and HOWE, M. (1938). Lipids of wheat flour. I. The petroleum ether extract. *Cereal Chem.* 15:716–720.

SULLIVAN, B., and NEAR, C. (1933). Change of crude lipides of wheat on storage. *Ind. Eng. Chem.* 25:100–103.

——, ——, and FOLEY, G. H. (1936). The role of the lipids in relation to flour quality. *Cereal Chem.* 13:318–331.

SUZUKI, U., YOSHIMURA, K., and TAKAISHI, M. (1907). Über ein Enzym "Phytase" das "Anhydro-oxy-methylen-diphosphorsäure" spaltet. *Tokyo Imp. Univ. Coll. Agr. Bull.* 7:495–503.

UZAWA, S. (1932). The phosphatases of bran, I and II. *J. Biochem.* (*Japan*) 15:1–10; 11–17.

VORBRODT, W. (1910). Untersuchungen über die Phosphoverbindungen in den Pflanzensamen mit besonderer Berücksichtigung des Phytans. *Bull. intern. acad. sci. Cracovie A*, 414–511.

WESTENBRINK, H. G. K., VAN DORP, D. A., GRUBER, M., and VELDMAN, H. (1940). Aneurinphosphates and yeast phosphatase. *Enzymologia* 9:73–89.

WIDDOWSON, E. M. (1941). Phytic acid and the preparation of food. *Nature* 148: 219–220.

WILLSTÄTTER, R., and MEMMEN, F. (1923). Zur stalagmometrischen Bestimmung der lipatischen Tributyrinhydrolyse. *Z. physiol. Chem.* 129:1–25.

—— and WALDSCHMIDT-LEITZ, E. (1924). Über Ricinuslipase. *Z. physiol. Chem.* 134:161–223.

ZELENY, L. (1940). Fat acidity in relation to heating of corn in storage. *Cereal Chem.* 17:29–37.

—— and COLEMAN, D. A. (1938). Acidity in cereals and cereal products, its determination and significance. *Cereal Chem.* 15:580–595.

—— and —— (1939). The chemical determination of soundness in corn. *U. S. Dept. Agr. Tech. Bull.* No. 644.

CHAPTER VI

OXIDIZING ENZYME SYSTEMS

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INTRODUCTION

The great chemist Lavoisier, who discovered the importance of oxygen in combustion and in respiration, defined oxidation in terms of the participation of oxygen. Higher and higher states of oxidation were related to the progressive action of oxygen. The reverse process, looked upon as leading to lower states—to reduced states—was called reduction. The term "reduction," however, was applied not only to the removal of oxygen but also to the addition of hydrogen. A time-consuming and wasteful controversy took place among biologists when Wieland (1932) attempted to displace oxygen from its time-honored position by emphasizing that most biological oxidations were actually dehydrogenations. This controversy, which raged mostly in Europe, was ignored in the United States undoubtedly because of the wise counsels of W. M. Clark. In fact, Clark and Cohen, in 1923, gave the following expression to a definition of oxidation that had had a long evolution: "The withdrawal of electrons from a substance with or without the addition of oxygen or elements analogous to oxygen; or . . . the withdrawal of electrons with or without the withdrawal of hydrogen or elements analogous to hydrogen." This all-embracing definition, adherence to which would have made the nomenclature of oxidases less bewildering and confusing, will be strictly followed in this chapter. The oxidation-reduction enzyme systems will be designated "oxidases" because this is the oldest term.

Foodstuffs, which are oxidized *in vitro* only by powerful oxidants or under conditions which do not exist in biological systems, are oxidized in living cells at relatively low temperatures, and at neutrality, through the action of mild oxidizing agents, the oxidation-reduction enzyme systems or oxidases. These enzymes occupy an all-important place for they provide

the energy required for the performance of life processes. Foodstuff is burned to carbon dioxide and water through a series of enzymatic reactions, some of them reversible, some irreversible, which transfer the electrons from the oxidizable substances to molecular oxygen by smooth steps. This stepwise electron transfer contributes to the complexity of oxidases. In no case is there direct electron transfer from oxidizable substrate to molecular oxygen. In the simplest oxidase, the substrate is oxidized by the enzyme, and the reduced enzyme is oxidized by molecular oxygen. In others, a number of oxidation-reduction systems are interposed between substrate and molecular oxygen. In such cases the oxidation reactions proceed in a series, the system of more negative potential being oxidized by the reduction of the system of more positive potential, and so on, in a series of graded steps up to the reduction of molecular oxygen. So, like locks in a canal, these reversible systems gradually release the oxidation energy.

Oxidases are, in general, made up of a specific protein, the *activating protein* or dehydrogenase, and a series of *oxidation-reduction systems* or coenzymes. Brief general discussions of these two components, which will facilitate the understanding of the different types of oxidases, are presented first. Oxidation-reduction systems of biological importance are then considered in greater detail, and a final section deals with the classification of the complete oxidase systems.

ACTIVATING PROTEINS

Oxidizable substrates, in combination with a specific protein molecule, the *activating protein,** become activated; that is, a change in the electronic structure of the substrate occurs by which the electrons of the oxidizable substrate become ready for transfer to molecular oxygen through the mediation of the oxidation-reduction components of the system. Substrate activation may be interpreted with Michaelis (1940) as an increase in the radical formation constant of the oxidizable substrate. Activation must then be taken as synonymous with radical formation. The activating protein combines reversibly with the oxidizable substrate, as first postulated by Michaelis and Menten (1913).

$$\text{Protein} + \text{substrate} \rightleftharpoons \text{protein–substrate}$$

The dissociation constants of these complex compounds are therefore determined by a simple application of the law of mass action.

* The activating protein is also called dehydrogenase, dehydrase, apodehydrogenase, *Zwischenferment.* The name dehydrogenase, introduced by Wieland and Thunberg at a time when the oxidation-reduction components of oxidases were unknown, is a misnomer because the activating protein does not "dehydrogenate" the oxidizable substrate. Dehydrogenation is effected by the oxidation-reduction systems (pyridine nucleotides, flavins, etc.).

$$K = \frac{\text{(protein) (substrate)}}{\text{(protein–substrate)}}$$

where "protein–substrate" is measured by the degree of enzyme activity. The manner of this combination—the groups on the protein molecule to which the substrate attaches itself—is unknown.

Of all the components of oxidation-reduction enzyme systems, the activating protein is the only one possessing remarkable specificity in its enzymatic action. It may have common properties when derived from different sources such as plants or animal tissues; or quite different proteins may be required. For example, the activating protein of yeast alcohol oxidase requires the presence of sulfhydryl groups for activity (Dixon, 1937), while that of liver alcohol oxidase does not (Lutwak-Mann, 1938).

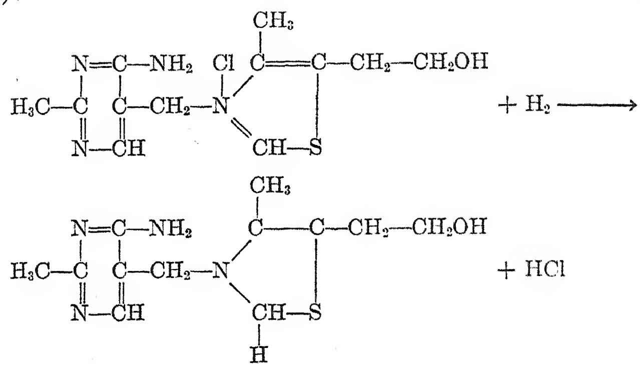
Very few of the activating proteins have been prepared in pure, crystalline form: the activating protein of yeast alcohol oxidase crystallized by Negelein and Wulff (1937); the activating protein of phosphoglyceraldehyde oxidase purified by Warburg and Christian (1939, 1939a); and the activating protein of lactate oxidase purified by Straub (1939). In general, the molecular weight of activating proteins is around 70,000. Denaturation by heat, acids, alkalies, or surface denaturation generally causes loss of enzyme activity. However, heat denaturation varies from protein to protein. Thus, the activating protein of lactic oxidase from gonococci is destroyed at 70° (Barron and Hastings, 1933), while the activating protein of liver choline oxidase is destroyed at 42°. The temperature coefficient of these reactions is around 2. The hydrogen-ion concentration is one of the most important factors controlling the reaction forming the substrate–protein complex, as shown by enzymatic activity measurements. In general, the optimum is between *p*H 7 and 8, and activity falls off asymptotically toward the acid and alkaline sides. Activity is lost at about *p*H 4 on the acid side and *p*H 10 on the alkaline side.

Inhibition experiments have shown that some groups in the protein molecule are essential for enzymatic activity. Among them, the sulfhydryl groups have been investigated most extensively. For this purpose mild oxidizing agents, mercaptide-forming compounds, and alkylating reagents were used. Enzyme inhibition by the first two reagents is reversible and reactivation can be brought about on addition of thiol compounds or other reducing agents. The experiments of Hopkins and co-workers (1938) on succinodehydrogenase can be presented as classical examples. Inhibition experiments have also shown that a large number of proteins require heavy metals as prosthetic groups: iron, magnesium, manganese, zinc, and cobalt. Denaturation of the protein with detergents or urethans also produces loss of activity, which shows that maintenance of the architectural integrity is essential for enzyme activity.

Diphosphothiamine–Proteins

Thiamine, or vitamin B_1, is a vitamin which acts as a component of enzyme systems. Many attempts have been made to show that thiamine is a reversible oxidation-reduction system, all of them ending in failure. It appears that its diphospho derivative should be classed as the prosthetic group of the activating protein of the enzymes concerned with the metabolism of α-keto acids, be it oxidation, dismutation, decarboxylation, or condensation.

Thiamine can be reduced by sodium dithionite (Lipmann and Perlmann, 1938):



Reduced thiamine is nonautoxidizable but is easily oxidized by hemochromogens (Barron and Lyman, 1941). However, neither the reduced nor the oxidized compound is physiologically active. Thiamine must be phosphorylated to act as a component of enzyme systems. This phosphorylation is performed enzymatically with adenosine triphosphate (ATP) acting as the phosphorus donor. In the enzymatic process (Lipschitz *et al.*, 1938), only as much diphosphothiamine is formed as is necessary to saturate the protein:

$$\text{Protein} + \text{ATP} \rightleftharpoons \text{protein–DPT} + \text{adenylic acid}$$

The reaction from right to left in this equation has not yet been demonstrated. Thiamine can be phosphorylated synthetically (Weijlard and Tauber, 1938). Phosphorylation takes place at the hydroxyl group of the thiazole nucleus of the molecule.

Enzyme Systems Containing Diphosphothiamine–Protein

Carboxylase* present in those cells which produce alcohol on glucose

* β-Carboxylase, the enzyme which decarboxylates oxalacetic acid to pyruvic acid and carboxylates pyruvate to oxalacetate, is not a thiamine–protein (Krampitz and Werkman, 1941).

fermentation (yeast, molds, plants) is a diphosphothiamine–protein (Lohmann and Schuster, 1937). The enzyme is protein–Mg–DPT (Kubowitz and Lüttgens, 1941; Green *et al.*, 1940) and contains one atom of magnesium and one molecule of diphosphothiamine (DPT) per mole of protein. The molecular weight of the enzyme is around 75,000 and it dissociates in mild alkaline solutions (*p*H 8) into protein and DPT. The enzyme decarboxylates α-keto acids into the corresponding aldehydes and carbon dioxide:

$$R\cdot COCOOH \longrightarrow RCOH + CO_2$$

The optimum hydrogen-ion concentration for the enzyme is around *p*H 6.2.

α-Ketoxidase (pyruvate oxidase) has DPT also in its activating protein. Activated pyruvate is oxidized by flavin dinucleotide in the presence of a phosphorus donor (Lipmann, 1939).

$$CH_3COCOOH + H_3PO_4 + \text{flavoprotein} \rightleftharpoons CH_3COOPO_3H_2 + CO_2 + \text{reduced flavin}$$

$$2\ CH_3COOPO_3H_2 + \text{adenylic acid} \longrightarrow 2\ CH_3COOH + ATP$$

Reduced flavin seems to be oxidized in three different ways: by atmospheric oxygen, by the cytochrome system, and possibly by fumarate (through reduction of fumarate to succinate).

α-Ketoglutarate oxidase is another DPT–protein (Barron, Gold-

TABLE I

ENZYME SYSTEMS CONTAINING DIPHOSPHOTHIAMINE–PROTEIN

Activated substrate	Nature of reaction	End-product determined	Reference
Pyruvate	Decarboxylation	Acetaldehyde + carbon dioxide	Lohmann and Schuster, 1937
Pyruvate	Oxidation	Acetate + carbon dioxide	Barron and Lyman, 1938; Lipmann, 1939
Pyruvate	Dismutation	Lactate + acetate + carbon dioxide	Barron and Lyman, 1938; Lipmann, 1939; Silverman and Werkman, 1939
Pyruvate	Dismutation	Acetate + formate	Barron and Jacobs, 1938
Pyruvate	Condensation	Acetylmethylcarbinol + carbon dioxide	Silverman and Werkman, 1941; Green, Westerfeld, Vennesland, and Knox, 1941
Pyruvate	Condensation	Carbohydrate	Barron, Lyman, Lipton, and Goldinger, 1941
Pyruvate	Condensation	Citrate	*Id.*
Pyruvate	Condensation	Acetoacetate	*Id.*
Pyruvate	Condensation	Succinate	*Id.*
Pyruvate	Condensation	α-Ketoglutarate	*Id.*
α-Ketoglutarate	Oxidation	Succinate	Barron, Goldinger, Lipton, and Lyman, 1941

inger, Lipton, and Lyman, 1941). This enzyme oxidizes α-ketoglutaric acid into succinic acid and carbon dioxide:

$$COOHCOCH_2CH_2COOH + \frac{1}{2}O_2 \longrightarrow COOHCH_2CH_2COOH + CO_2$$

It also requires a phosphorus donor (Ochoa, 1944).

Pyruvate mutase, the enzyme which produces dismutation of pyruvate into lactic acid and acetic acids, is also a DPT–protein:

$$2\,CH_3COCOOH + H_2O \longrightarrow CH_3CHOHCOOH + CH_3COOH + CO_2$$

Condensation reactions of pyruvate leading to the formation of acetyl methylcarbinol, α-ketoglutaric acid, citric acid, and carbohydrate also require the presence of DPT–proteins in some step of the reaction as yet undetermined. A summary of information on some reactions of pyruvate, and pertinent references, are given in Table I. All these reactions are irreversible.

OXIDATION-REDUCTION SYSTEMS

Thermodynamics. The oxidation-reduction systems which as components of oxidases transfer electrons from oxidizable substrate to molecular oxygen are thermodynamically reversible. The potentials of these systems can be derived from the classical equation developed first in 1898 by Peters:

$$E_h = E_0 - \frac{RT}{nF}\ln\frac{(\text{Red})}{(\text{Ox})} - \frac{RT}{F}\ln\frac{1}{(H^+)} - \frac{RT}{nF}\ln K$$

where E_h is the e.m.f. value referred to the hydrogen electrode, E_0 is a constant as defined by Clark and Cohen (1923a), R is the gas constant, T is the absolute temperature, F is the faraday, K is the dissociation constants of oxidant (Ox) and reductant (Red), and H^+ is the hydrogen-ion activity. At constant pH and when (Red)/(Ox) = 1, $E_h = E_0'$.

From these E_0 values, the free energy ΔF of the systems can be easily determined from the equation: $-\Delta F = nFE$, where n is the number of electrons involved in the reaction, F is the faraday, and E is the e.m.f. value.

For an isothermal process, the two quantities $-\Delta F$ and $-\Delta H$ are related to one another by the fundamental thermodynamic equation:

$$-\Delta F = \Delta H - T\Delta S$$

where ΔH is equal to the heat of reaction, T is the absolute temperature, and ΔS is the increase in entropy of the system under consideration.

The decrease in useful high-grade energy (equivalent to work), $-\Delta F$, in general plays by far the most useful role as a criterion for determining the

direction and extent of a chemical reaction. If ΔF has a large negative value, the process involved *may* take place spontaneously at constant temperature and pressure; if ΔF is positive for a process, there must be an increase of free energy in the system and the process will take place only through the application of work; if ΔF is zero, a state of equilibrium exists and no further change is to be expected.

Of the oxidation-reduction systems which act as components of oxidases, some (metal–porphyrins, metal–proteins) pass from the state of oxidation to that of reduction by the transfer of one electron; others (flavins, pyridine nucleotides, plant pigments), by the transfer of two electrons. However, in these last systems (Michaelis, 1937), there may be three different levels of oxidation: the totally oxidized form T; the intermediate (semioxidized) form S; and the reduced form R:

$$R \rightleftharpoons T + 2e$$

This process, if separated into two steps, becomes:

$$(1) \quad R \rightleftharpoons S + e$$

$$(2) \quad S \rightleftharpoons T + e$$

The equilibrium being conditioned by the reversible process:

$$R + T \rightleftharpoons 2S$$

The equilibrium constant is:

$$\frac{(S)^2}{(R)(T)} = k = \frac{1}{K}$$

where k is the *radical formation constant* or *semiquinone formation constant* and K is the *dismutation constant.* The value of the radical formation constant may vary greatly even for one substance with change of hydrogen-ion concentration, etc. Free radicals exist in all the systems of biological importance hitherto considered as passing from oxidation to reduction through transfer of two electrons. According to Michaelis, this passage from oxidation to reduction *always* proceeds by the transfer of *one electron* through radical formation. Provided the radical formation constant is not too small, its concentration is not the limiting factor for the rate of reaction. If, however, this constant becomes too small, the concentration of the radical may become the limiting factor of the rate, and the process will be sluggish.

According to this theory, in the enzyme–substrate complex the equilibrium between the oxidized form of the substrate, the reduced form, and the intermediate radical would be more in favor of the radical than in

the uncombined substrate itself. This hypothesis provides a satisfactory explanation of those instances in which the rate of biological oxidations is proportional to the oxidation-reduction potential of the oxidizing catalyst, *i.e.*, the free energy of the system (Barron, 1932). In fact, theories of reaction rates developed by La Mer (1933), Eyring (1935), and Rice (1936) make explicit use of an activated complex. The specific rate constant, k_i, of these reactions depends on the free energies of the activated systems:

$$k_i = \exp - \frac{\Delta F^*}{RT} - \frac{kT}{h}$$

where $-\Delta F^*$ is the free energy of formation of the activated stage, and k and h are the Boltzmann and Plank constants, respectively. By combination of the oxidizable substrate with the activating protein, the energy of formation of the activated state $-\Delta F^*$ would become equal to the energy of the reaction $-\Delta F$, and the rate of reaction would be determined by the oxidation-reduction potential.

The value $-\Delta F$ is useful not only for determining whether an oxidation process is thermodynamically possible, and for predicting rates of reactions, but also for giving us the useful energy of the reaction, *i.e.*, that portion of $-\Delta H$ (the total heat of the reaction) which can be used directly for work.

Classification of Oxidation-Reduction Systems. It is known that reversible oxidation-reduction systems behave differently toward the sluggish universal oxidant, atmospheric oxygen. Some of them are readily oxidized; others are not attacked by it. These differences are of great importance in the understanding of the mechanism of biological oxidations. From the biological point of view these reversible oxidation-reduction systems can be divided into three groups: electromotively active systems, sluggish systems, and enzymatic-sluggish systems.

Electromotively active systems can exchange electrons directly with a noble metal electrode, and their free energies can be determined by the usual potentiometric methods so well described by Clark and Cohen (1923a). As a rule, they are autoxidizable, that is, readily oxidized by atmospheric oxygen.

Sluggish systems, although thermodynamically reversible, are electromotively sluggish; their potentials cannot be satisfactorily measured except with the aid of electroactive mediators. These potentials can then be measured potentiometrically, colorimetrically, or spectrophotometrically, provided the sluggish system or the electroactive mediator can be determined by these methods. For example, the potential of cysteine was measured colorimetrically by Fruton and Clarke (1934) with the aid of reversible dyes; the potential of cytochrome c was measured spectrophotometrically

by Stotz *et al.* (1938) with the aid of dyes and quinone. Sluggish systems are, as a rule, nonautoxidizable, that is, they are not oxidized by atmospheric oxygen at a measurable rate and within the hydrogen-ion range found in physiologic conditions. They are easily oxidized by electromotively active substances, and easily reduced by a number of oxidizable substances.

Enzymatic-sluggish systems are those in which equilibrium is attained in the presence of an enzyme and an electroactive system. Determination of the potentials of these reversible systems thus requires the mediation of two factors: the enzyme, which acts as a perfect catalyst; and the electroactive system, which is used to measure the potential of the system. These potentials may be measured either colorimetrically or potentiometrically. In fact, the first measurements of the potentials of this type of system were performed colorimetrically by Quastel and Wooldridge (1928). They measured the potential of the system, succinate $- 2\,e \rightleftharpoons$ fumarate $+ 2\,H^+$, with the aid of methylene blue.

As will be seen later, this classification of the oxidation-reduction systems is of practical biological utility; for, as a rule, the sluggish systems are the direct oxidizing agents of the oxidizable substrates, while the electroactive systems transfer electrons from the sluggish systems to molecular oxygen. Why some of these thermodynamically reversible systems are sluggish and some are electroactive is not yet known, although the elucidation of this property is of great biological significance.

INDIVIDUAL OXIDATION-REDUCTION SYSTEMS OF BIOLOGICAL IMPORTANCE

It is most useful to discuss the principal oxidation-reduction systems under the classification given in the preceding section. However, one important class of compounds, the iron–porphyrins, is discussed separately because they form both sluggish and electromotively active systems.

The Iron–Porphyrin Compounds

The iron–porphyrin compounds occupy a unique position among the oxidation-reduction systems of biological importance, because, on combining with nitrogenous compounds, they give complexes possessing a variety of properties, all of them connected with the function of respiration. Thus, when certain iron–porphyrins combine with a protein they form compounds (hemoglobin, myoglobin, erythrocruorin) which can combine reversibly with molecular oxygen, with no electron exchange. They act as oxygen storehouses in animal multicellular organisms, and do not exist in plants. Iron–porphyrins combine with proteins to form sluggish reversible oxidation-

reduction systems (cytochrome *c*) which are not oxidized by molecular oxygen and act as intermediate electron transfer catalysts, which transfer electrons from one reversible system to another. Iron–porphyrins combine with proteins or with nitrogenous bases to form electromotively active reversible systems [cytochrome *b* (?), cytochrome oxidase (?), hemochromogens]. The property of iron–porphyrins and of their nitrogenous coordination compounds of possessing strong absorption bands, and the property of some

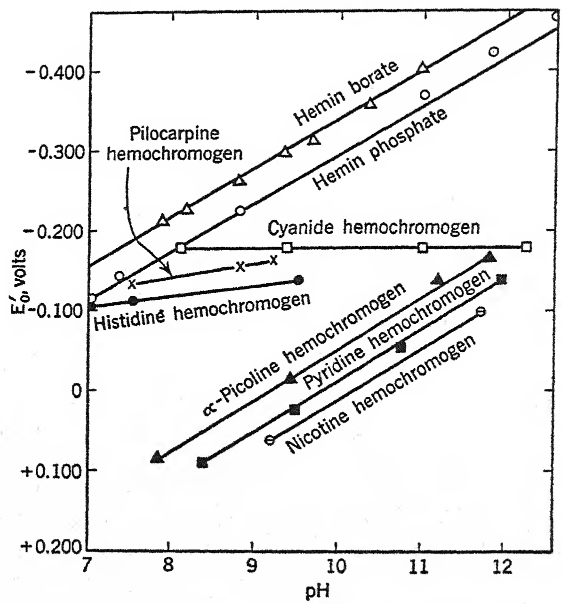


Fig. 1.—The relation of hydrogen-ion concentration (pH) to the E_0' values of hemin and of its hemochromogens (Barron, 1937).

of these compounds of combining reversibly with hydrogen cyanide and carbon monoxide have both been extensively used for their detection in living cells. Hydrogen cyanide combines with the ferriporphyrins; carbon monoxide combines with the ferroporphyrins.

The reversibility of the iron–porphyrin nitrogenous compounds was discovered in 1925 by Anson and Mirsky (1925):

$$Fe^{+++} + Nn \rightleftharpoons Fe^{+++}Nn; \quad Fe^{++} + Nm \rightleftharpoons Fe^{++}Nm$$

where N represents a nitrogenous substance, and n and m the number of moles combining with iron–porphyrin. This discovery was of first importance for the understanding of the properties of these substances.

Conant and his collaborators (1928, 1930) were the first to demonstrate that iron–protoporphyrins and their nitrogenous derivatives are reversible oxidation-reduction systems. These investigations were extended by Barron (1937), who studied the behavior of a number of hemochromogens (iron–porphyrin–nitrogenous derivatives) and the oxidation-reduction potentials (Fig. 1). Clark and his co-workers continued these studies and gave a clear quantitative treatment of the empirical findings (Clark *et al.*, 1940; Clark and Perkins, 1940).

Cytochromes. Of the iron–porphyrin–protein compounds, the cytochromes, discovered in 1887 by MacMunn and rediscovered in 1925 by Keilin, deserve special mention because of their role as oxidation-reduction catalysts and because of their universal distribution. Cytochromes are present in animal tissues, plants, and bacteria. Their detection was made easy because of their striking absorption spectra. Three cytochromes were named by Keilin, *a*, *b*, and *c*, characterized by the position of the absorption bands in the visible portion of the spectrum (Table II).

TABLE II

ABSORPTION SPECTRUM BANDS OF SOME PORPHYRINS AND IRON–PORPHYRIN COMPOUNDS

Compound	Absorption bands				
	I	II	III	IV	V
	Å	Å	Å	Å	Å
Etioporphyrin III	6227	5665	5264	4944	4000
Protoporphyrin	6296–6048	5786	5353	5010	4000
Fe^{+++}–protoporphyrin (ferrihemin)	6100	—	—	4925	3850
Fe^{++}–protoporphyrin (ferrohemin)	—	5660	5370	—	4100
Cyanide ferriprotoporphyrin	—	5450	—	—	4255
Cyanide ferroprotoporphyrin	—	5660	5370	—	4330
Spirographisporphyrin	6430–5925	5810	5550	5145	?
Cyanide ferrispirographisporphyrin	—	—	5500	—	4290
Cyanide ferrospirographisporphyrin	—	5950	5400	—	4480
Cytochrome *c* (ferro)	—	—	5500	5200	4150
Cytochrome system in bakers' yeast	6030	5650	5500	5230	4490, 4330, 4170
Cytochrome system in brewers' yeast	5860	5650	5510	5250	4490, 4330, 4170
Cytochrome system in beewing muscles	6048	5665	5502	5210	4490, 4330, 4170
Cytochrome system in *E. coli*	6280	5900	5600	5300	—
Cytochrome system in *B. azotobacter*	6320	5890	5630–5510	5260	4300
Catalase	6270	—	5360	5020	4090
Peroxidase I	—	5750	5400	4200	4150, 4200
Peroxidase II	6400	—	5480	—	—
Verdoperoxidase, Fe^{+++}	6900–6250	5700	—	5000	—
Verdoperoxidase, Fe^{++}	6370	—	5450	—	—
CO–cytochrome oxidase, heart	—	5890	—	5100	4500

(1) Cytochrome *a* has, in the reduced state, two absorption bands: 6000–6050 Å and 5200 Å. According to Keilin and Hartree (1940), it does not combine with carbon monoxide or hydrogen cyanide. The E_0' value at *p*H 7 is about +0.29 volt (Ball, 1938). Yakushiji and Okunuki (1941) claim to have isolated cytochrome *a* from the heart muscle by extraction with sodium cholate and alkaline phosphate. The green, reduced compound had a strong absorption band at 6050 Å and a weak band at 5130 Å, carbon monoxide having no effect on the spectrum. Reduced cytochrome *c* was partially oxidized by the oxidized cytochrome *a*.

(2) Cytochrome *b* has, in the reduced state, two absorption bands: 5640 Å and 5300 Å. It is believed to be an autoxidizable substance, that is, easily oxidized by atmospheric oxygen. Cytochrome *b* would therefore be an electroactive oxidation-reduction system like the hemochromogens. Cytochrome *b* seems to be thermolabile and is closely bound to the insoluble material in tissue extracts. Yakushiji and Mori (1937) claim to have isolated it in soluble form. It does not combine with hydrogen cyanide, carbon monoxide, or other respiratory inhibitors. The E_0' value, *p*H 7.0, was estimated by Ball (1938) to be in the neighborhood of −0.04 volt.

(3) Cytochrome *c* is the only component which has been isolated and prepared in pure form (Theorell, 1936; Keilin and Hartree, 1937). The chemical and physical properties have been thoroughly studied by Theorell and Åkesson (1941). The molecular weight of this iron–porphyrin–protein is quite low, 13,000. The iron content is 0.43 per cent. The protein moiety contains 31 to 32 free amino groups of which 22 belong to lysine. Among the amino acids isolated in pure state from cytochrome *c* are cysteine, lysine, tyrosine, leucine, and glutamic and aspartic acids. The absorption spectrum of the ferri form shows not less than five different absorption lines. None of these five types of ferricytochrome *c* combines with hydrogen cyanide—contrary to the claims of Potter (1941)—unless in very alkaline solutions of not lower than *p*H 13. Between *p*H 3 and 10, cytochrome *c* does not combine with carbon monoxide, contrary to the claim of Altschul and Hogness (1938). See H. Theorell and Å. Åkesson, *J. Am. Chem. Soc.*, **63:** 1812 (1932) for experimental evidence. This substance is remarkable for its stability. It is not affected by 0.1 *M* hydrochloric acid or 0.1 *M* sodium hydroxide and can be heated to boiling temperature. Cytochrome *c* is—at values between *p*H 4 and 10—a sluggish oxidation-reduction system; it is not oxidizable by molecular oxygen. The oxidation-reduction potential of this system, like that of cyanide hemochromogen, is independent of the hydrogen-ion concentration between values of *p*H 5 and 8 where the E_0' value is +0.26 volt (Wurmser *et al.*, 1938; Stotz *et al.*, 1938). Cytochrome *c* is easily reduced by a variety of agents, such as hydrosulfite, ascorbic acid, cysteine, adrenaline, hydroquinone, and *p*-

phenylenediamine. It is reduced by reduced alloxazine mononucleotide–protein, the cytochrome reductase of Haas *et al.* (1940).

(4) Cytochrome oxidase oxidizes the nonautoxidizable cytochromes, and is, in its turn, oxidized by molecular oxygen. Cytochrome oxidase is thus the last link in the stepwise oxidation of most foodstuffs. An example of how these last reactions proceed may be given by following the reduction of cytochrome *c* ($Fe_{cyt.}{}^{+++}$) by the reduced flavoprotein (FH_2) "cytochrome reductase"; the oxidation of reduced cytochrome *c* by cytochrome oxidase ($Fe_{cyt.ox.}{}^{+++}$); and the oxidation of reduced cytochrome oxidase by oxygen:

$$FH_2 + 2\,Fe_{cyt.}{}^{+++} \rightleftharpoons 2\,Fe_{cyt.}{}^{++} + F + 2\,H^+$$

$$2\,Fe_{cyt.}{}^{++} + 2\,Fe_{cyt.ox.}{}^{+++} \rightleftharpoons 2\,Fe_{cyt.}{}^{+++} + 2\,Fe_{cyt.ox.}{}^{++}$$

$$2\,Fe_{cyt.ox.}{}^{++} + O_2 \rightleftharpoons 2\,Fe_{cyt.ox.}{}^{+++} + 2\,O^-$$

$$2\,O^- + 2\,H^+ \longrightarrow H_2O_2;\ H_2O_2 \longrightarrow H_2O + {}^1/_2\,O_2$$

The hydrogen peroxide is catalytically destroyed by catalase according to reactions which will be described later. The rate of oxidation of these systems is practically independent of the partial pressure of oxygen. Cytochrome oxidase is extremely sensitive to the action of hydrogen cyanide, hydrogen sulfide, and sodium azide. It forms with carbon monoxide a reversible compound which is dissociated by light. It was indeed Warburg's discovery (1926) of carbon monoxide inhibition of yeast respiration, and of abolition of this inhibition by monochromatic light, which led him to the determination of the relative absorption spectrum of this *Sauerstoffübertragendes Ferment* (1932) and the establishment of iron–porphyrin as the prosthetic group of the enzyme (Fig. 2, page 188). From the position of the absorption bands Warburg concluded that the iron–porphyrin was similar to pheohemin compounds (green hemins).

Cytochrome oxidase, for many years considered firmly attached to the insoluble portion of cell structures, has been brought into solution by Haas (1943) by the aid of prolonged grinding and autolysis and by ultrasonic waves. It seems that Haas's cytochrome oxidase is made up of two components: component I, which loses 80 per cent of its activity in 15 minutes at 50°; and component II, which may be kept for the same length of time in boiling water without loss of activity (Haas, 1944).

Cytochromes play an important role in biological oxidations. Their fundamental position is shown by the inhibition of respiration with hydrogen cyanide, and by the observation of Haas (1934) that the rate of alternate oxidation and reduction of cytochrome *c* in intact yeast cells could account for all the oxygen consumption of yeast. Apart from the plausible assumption—plausible because of the highly positive oxidation-reduction potential of these systems—that the cytochrome system is the last link in

the stepwise electron transfer from oxidizable substrate to molecular oxygen, little is known of the properties of these systems, and little will be known until they are isolated and their chemical properties determined. Is there only one cytochrome between cytochrome oxidase and the other oxidation-reduction steps in the chain of oxidation-reduction systems present in oxidases? Are the electrons transferred through all the cytochromes to cytochrome oxidase: cytochrome *b* → cytochrome *c* → cytochrome *a* → cytochrome oxidase?

Cytochrome *c* is rapidly reduced by a flavoprotein (alloxazine mononucleotide) isolated by Haas and co-workers (1940). The necessity of the

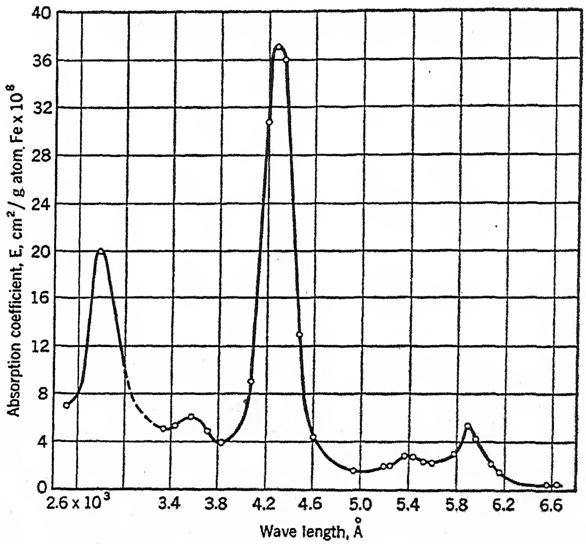


Fig. 2.—The absorption spectrum of CO–cytochrome oxidase of yeast.

electroactive mediator for the transfer of electrons to cytochrome *c* is a consequence of the sluggishness of this system. That another flavoprotein is required for the stepwise electron transfer through cytochrome *b* is possible, though not as yet demonstrated. Many oxidases require the presence of the cytochrome systems: succinoxidase, choline oxidase, α-hydroxyoxidase (lactate), pyruvate oxidase, glycerol oxidase, etc. Yet some of these oxidases, when found in cytochrome-lacking cells, do not require cytochromes to perform the oxidations. For example, α-hydroxyoxidase contains cytochrome oxidase in the system in animal tissues and cytochrome-containing bacteria (inhibition by carbon monoxide and cyanide); it does not require cytochromes in cells lacking cytochrome. The same

holds for pyruvate and glycerol oxidases. It may then be concluded that the cytochrome system (the last link in the stepwise transfer of electrons from oxidizable system to molecular oxygen) is, as a rule, a component of a large number of oxidases in those cells possessing the cytochrome system. In cells lacking cytochromes, transfer of electrons is performed *via* flavins to molecular oxygen. In cells *in the resting state*, transfer of electrons might be effected also without the mediation of the cytochrome system.

Catalase. This iron–porphyrin–protein compound was prepared for the first time in crystalline form by Sumner and Dounce (1937). The molecular weight is 225,000 (Sumner *et al.*, 1941). Catalase decomposes hydrogen peroxide, presumably according to the following reaction (Keilin and Hartree, 1938), in which hydrogen peroxide is reduced by the ferri compound and reduced catalase is reoxidized by molecular oxygen:

$$4\,Fe^{+++} + 2\,H_2O_2 \longrightarrow 4\,Fe^{++} + 4\,H^+ + 2\,O_2$$

$$4\,Fe^{++} \longrightarrow 4\,H^+ + O_2 \longrightarrow 4\,Fe^{+++} + 2\,H_2O$$

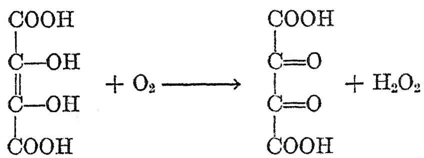
Some investigators have, however, challenged the existence of these reactions (Johnson and van Schouwenburg, 1939; Weiss and Weil-Malherbe, 1939).

Crystalline catalase has been prepared from liver and from beef erythrocytes. Catalase is one of the most active enzymes. One molecule can decompose 2.6×10^6 molecules of hydrogen peroxide per minute. The optimum temperature lies between 0° and 10°C, presumably because of the rapid destruction of catalase by hydrogen peroxide at higher temperatures. Catalase is rather unstable in acid solutions and is destroyed at about *p*H 3. It is inactivated by hydrogen cyanide, hydrogen sulfide, hydroxylamine, and sodium azide.

Peroxidase. This iron–porphyrin–protein enzyme is present in nearly all plants. It has been highly purified by Theorell (1940), who separated two distinct enzymes from horse-radish peroxidase. Peroxidase I is a rather unstable enzyme, not always present in horse-radish, which has absorption bands at 5750, 5400, 5200, and 3700 Å. Peroxidase II, the common horse-radish enzyme, was obtained in crystalline form (1942). The molecular weight is 44,000 and there is one mole of iron–porphyrin per mole of protein. Peroxidase gives three different compounds with hydrogen peroxide (Theorell, 1942a): a greenish compound with one strong absorption band at 6550 to 6600 Å; and two red compounds with two absorption bands, at 5600 and 5300 Å, and at 5830 and 5450 Å, respectively. The green compound, which forms with low concentrations, is the only one catalytically active. With increases of the hydrogen peroxide concentration a new apparently inactive compound forms, which explains the enzyme inhibition when the hydrogen peroxide concentration is high.

Peroxidase in the presence of hydrogen peroxide catalyzes the oxidation of a very large number of phenols and aromatic amines. It oxidizes iodides, setting free iodine. Peroxidase is less sensitive to high temperatures than many other enzymes. Even after it is boiled, the preparation may become active again if it is allowed to stand for several hours. Peroxidase is inactivated by an excess of hydrogen peroxide, but the activity returns when this excess is removed. Hydrogen cyanide, hydrogen sulfide, sodium azide, nitric oxide, hydroxylamine, sodium hydrosulfite, and thiourea inhibit the enzyme. It has been postulated that peroxidase plays an important role in plant respiration, but evidence for this claim is lacking.

The enzyme dihydroxymaleic acid oxidase, discovered by Banga *et al.* (1938, 1939), is present in plants, and oxidizes dihydroxymaleic acid to diketosuccinic acid:



It has been shown by Theorell and Swedin (1939) to be identical with peroxidase. According to them, dihydroxymaleic acid oxidizes spontaneously with formation of hydrogen peroxide. On addition of peroxidase, the enzyme–peroxidase complex oxidizes more of the substrate. The addition of catalase prevents the action of peroxidase through destruction of the peroxide.

Verdoperoxidase. This enzyme, which occurs in leucocytes, has been purified by Agner (1941). The enzyme is brownish green when oxidized and green when reduced. In contrast to other peroxidases, the prosthetic group of this enzyme cannot be split from the protein by acetone–hydrochloric acid. The enzyme forms compounds with hydrogen peroxide, hydrogen cyanide, sodium azide, and hydroxylamine, but not with carbon monoxide or sodium fluoride.

Electroactive Systems

In the chain of stepwise oxidations which occur in the living cell, and sometimes even in single isolated enzyme systems, the electroactive systems play an important role as the mediators of electrons either between oxidizable substrate and molecular oxygen or between sluggish systems. An example of the first type is flavin dinucleotide in *d*-amino acid oxidase, and of the second type, Haas's cytochrome reductase, where flavin mediates electron transfer between triphosphopyridine nucleotide and cytochrome *c*.

Flavoproteins. Among the electroactive oxidation-reduction systems present in biologic systems (Table III), riboflavin is the most widely distributed in nature. It is present in free form or as the phosphate derivative in combination with proteins, in animal tissues, plants, yeasts, and bacteria. Riboflavin was obtained from heart muscle by Banga *et al.* (1932), and from

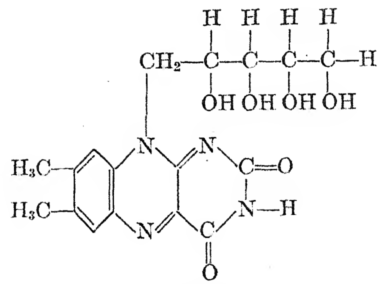
TABLE III

OXIDATION-REDUCTION POTENTIALS OF ELECTROACTIVE SYSTEMS PRESENT IN BIOLOGICAL FLUIDS

Substance	Origin	E_0' at pH 7.0	Temp.	Reference
		volts	°C	
Adrenalone	Animal	+0.489	30	Ball, Chen, and Clark, 1933
Epinephrine	Animal	+0.389	30	*Id.*
Homogentisic acid	Animal	+0.260	30	Fishberg and Dolin, 1933
Urechrome	Animal	+0.186	30	Horowitz and Baumberger, 1941
1,4-Naphthoquinone	Plant	+0.064	20	Fieser and Fieser, 1935
Phoenicein	Mold	+0.047	20	Friedheim, 1933
Juglone	Plant	+0.036	20	*Id.*, 1934
Pigment from *Arion rufus*	Plant	+0.025	20	*Id.*, 1932
Hallachrome	Animal	+0.022	20	*Id.*, 1933a
2-Methyl-1,4-naphthoquinone	Plant	−0.012	20	Fieser and Fieser, 1935
Pyocyanine	Bacteria	−0.034	30	Michaelis and Friedheim, 1931; Elema, 1931
Hermidin	Plant	−0.030		Cannan, 1926
Toxoflavin	Bacteria	−0.049		Stern, 1935
Vitamin K	Plant	−0.057	20	Riegel *et al.*, 1940
Phosphoriboflavin–protein	Yeast	−0.061	38	Kuhn and Boulanger, 1936
Flavin-dinucleotide-protein (xanthine oxidase)	Liver	−0.080 −0.090	30	Ball, 1939
Chromodoris zebra pigment	Plant	−0.102	30	Preisler, 1930
Chlororaphin	Bacteria	−0.115	30	Elema, 1933
Lawsone	Plant	−0.139	20	Friedheim, 1934
Phthiocol	Bacteria	−0.179	30	Ball, 1934; Hill, 1936
Lapachol	Plant	−0.180	30	Ball, 1936; Hill, 1938
Lomatiol	Plant	−0.184	30	Ball, 1936; Hill, 1938
Riboflavin	Animal and vegetable	−0.208	30	Michaelis, Schubert, and Smythe, 1936
Echinochrome	Animal	−0.221	30	Cannan, 1927
Flavin–dinucleotide	Liver	−0.226	30	Ball, 1939a

brewers' yeast by Warburg and Christian (1932), who described the flavin–protein complex as yellow enzymes. György, Kuhn, and Wagner-Jauregg (1936) showed that riboflavin is identical with vitamin B_2. It is 6,7-dimethyl-9-(1-*d*-ribityl)-isoalloxazine.

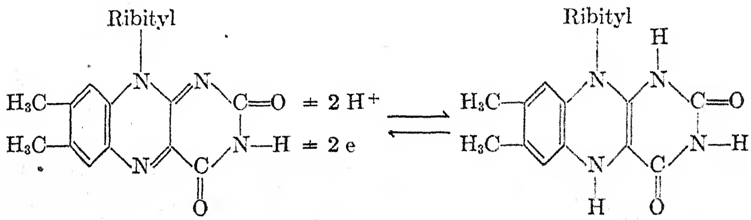
Riboflavin was synthesized by Kuhn *et al.* (1935) and by Karrer *et al.* (1935). Riboflavin phosphate can be synthesized by treating riboflavin with phosphoryl chloride (Kuhn and Rudy, 1935). It can be phosphorylated by adding intestinal epithelial powder to a solution of riboflavin in



Riboflavin

phosphate buffer (Pulver and Verzár, 1939). Riboflavin in aqueous solutions is yellow with a green fluorescence. When irradiated in vacuum with visible light, it loses the yellow color; on admission of air, the color reappears and deuteroriboflavin is formed. Lumiflavin is formed on addition of strong alkali; lumiflavin is soluble in chloroform and insoluble in water, and its solution is yellow with a green fluorescence.

Riboflavin is a reversible oxidation-reduction system:



In neutral solutions riboflavin becomes colorless on reduction. In acid solutions formation of a radical, semiquinone, of red color, occurs so that reduction proceeds: yellow ⇌ red ⇌ colorless. According to Michaelis and co-workers (1936) riboflavin gives this radical at all hydrogen-ion concentrations, the maximum amount of semiquinone formed at hydrogen-ion concentrations of physiological significance being about 10 per cent.

Phosphoriboflavin (mono- and dinucleotide) combines with proteins forming reversible flavoprotein compounds, which are important components of various enzyme systems (Table IV). This combination brings forth two changes in the oxidation-reduction properties of flavin, both of great biological significance: a change in the radical formation constant, so that at physiological hydrogen-ion concentrations there is ready radical formation (Haas, 1938); and a change in the oxidation-reduction potential. Flavin, like iron–porphyrins, increases the value of its oxidation-reduction potential on combination with protein. For example, the E_0' value of riboflavin at pH 7 goes up from -0.20 volt to -0.061 when it combines in yeast with a protein.

TABLE IV

THE FLAVOPROTEINS AS COMPONENTS OF ENZYME SYSTEMS

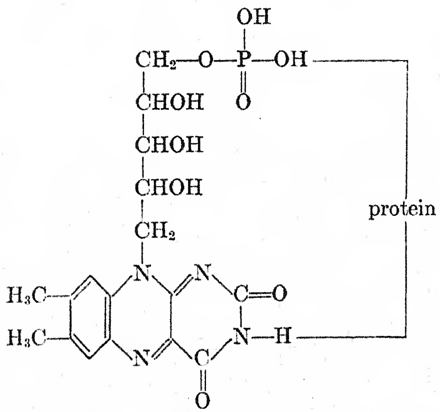
Property of protein	Flavin	Reductant	Oxidant	Reference
Activating protein (*d*-amino acid oxidase)	Dinucleotide	*d*-Amino acids	Oxygen	Warburg and Christian, 1938a
Activating protein (xanthine oxidase)	Dinucleotide	Xanthine, other purines, aldehydes	Oxygen	Ball, 1939
Activating protein (aldehyde oxidase)	Dinucleotide	Aldehydes	Unknown electro-active system	Gordon, Green, and Subrahmanyan, 1940
Electron mediator (cytochrome *c* reductase)	Mononucleotide	TPN	Cytochrome *c*	Haas, Horecker, and Hogness, 1940
Electron mediator	Dinucleotide	DPN	Cytochrome *b* (?)	Haas, 1938; Straub, 1939
Activating protein (fumaric dehydrogenase)	Dinucleotide	Unknown	Fumarate	Fischer and Eysenbach, 1937; Fischer, Roedig, and Rauch, 1939
Activating protein (pyruvate oxidase)	Dinucleotide	Pyruvate	Fumarate (?) or oxygen	Lipmann, 1939

The formulas of flavin mononucleotide (I) and dinucleotide (II) may be presented thus:

(I) Isoalloxazine-*d*-ribose-phosphate

(II) Isoalloxazine-*d*-ribose-phosphate-phosphate-*d*-ribose-adenine

Theorell (1937) studied the nature of the flavoprotein complex and demonstrated the reversibility of the reaction: flavin + protein $\rightleftharpoons$ flavoprotein. Each of the flavoproteins dissociates to a different extent into its prosthetic group and its protein moiety. According to Theorell, linkage of the phosphorylated flavin to protein occurs as follows:



The molecular weights of flavoproteins are around 70,000. Flavoproteins act as components of enzyme systems, that is, as electron mediators between sluggish systems or as the prosthetic group of enzyme systems.

The "cytochrome *c* reductase" of Haas *et al.* (1939, 1940) obtained from yeast is a flavin–mononucleotide–protein and acts as the electron me-

diator between reduced triphosphopyridine nucleotide [(TPN)H_2] and cytochrome *c:*

$$(TPN)H_2 + \text{flavin} \rightleftharpoons TPN + \text{flavin}\cdot H_2$$

$$\text{Flavin}\cdot H_2 + 2\,Fe^{+++} \rightleftharpoons \text{flavin} + 2\,Fe^{++} + 2\,H^+$$

The system used by Haas *et al.* (1940) in measuring the activity of this flavoprotein contains activating protein of hexosemonophosphate oxidase, hexose monophosphate, TPN, cytochrome *c*, and flavoprotein. The reduction of cytochrome *c* is followed spectrophotometrically at 5500 Å. The specific reaction velocities at 25° for these reactions are: 1.7×10^8 for the first and 53×10^8 for the second. The molecular weight of the flavoprotein is about 75,000, and the dissociation constant is 1×10^{-9}. As an autoxidizable system, this flavoprotein reacts with molecular oxygen, although the specific reaction velocity constant is quite small, 8×10^3.

Less is known about the flavin-dinucleotide–proteins which act as intermediate electron mediators, although a number of such flavoproteins have been reported by Dewan and Green (1937), Euler and Hellström (1938), Corran and Green (1938), Haas (1938), and Straub (1939). They seem to act as electron mediators between diphosphopyridine nucleotide and some other oxidation-reduction system. A flavoprotein discovered by Fischer *et al.* (1937, 1939) acts as mediator in the reduction of fumarate to succinate.

The flavoproteins do not possess the specificity of the activating proteins. Flavin dinucleotides may be combined with proteins other than the original protein. For example, a flavin from Euler's "diaphorase," on combining with the protein component of *d*-amino acid oxidase, acts as amino acid oxidase. Flavoproteins, although electroactive systems, act in cytochrome-containing cells as electron mediators from pyridine nucleotides to cytochromes. In cells lacking cytochrome, they act as the electroactive mediators with molecular oxygen.

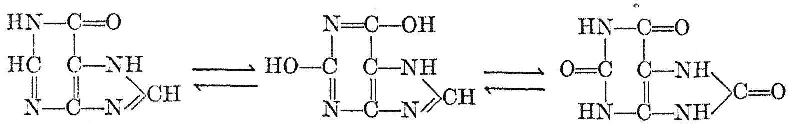
d-Amino acid oxidase and xanthine oxidase are examples of flavins acting as the prosthetic group of enzymes. The first was isolated by Warburg and Christian (1938a) and consists of a protein and alloxazine dinucleotide. The dissociation constant of the protein–flavin complex is 2.5×10^{-7}. The *d*-amino acid oxidase dissociates at *p*H 8.5, the optimum hydrogen-ion concentration for its activity, to the extent of 90 per cent. Although *d*-amino acid oxidase behaves as a dissociated flavoprotein in solution, it precipitates out of solution at neutrality as a conjugated flavoprotein. There is evidence that the reduced form of the oxidase does not dissociate. The enzyme catalyzes the oxidation of *d*-amino acids to the corresponding keto acids as follows:

$$R\text{—}\underset{\displaystyle NH_2}{\overset{\displaystyle H}{C}}\text{—}COOH + O_2 \longrightarrow R\text{—}C{=}NH\text{—}COOH + H_2O_2$$

$$R\text{—}C{=}NH\text{—}COOH + H_2O \longrightarrow R\text{—}C{=}O\text{—}COOH + NH_3$$

The second reaction is spontaneous.

Xanthine oxidase, which was purified by Ball (1939), is also a flavoprotein; the prosthetic group is alloxazine dinucleotide (Warburg and Christian, 1938, 1938a). It has little specificity towards purines. It oxidizes hypoxanthine and xanthine and seven other purines.



Hypoxanthine Xanthine Uric acid

These two steps are reversible, as demonstrated by Filitti (1935) and by Green (1934). Xanthine oxidase also oxidizes a variety of aliphatic and aromatic aldehydes.

Other Electroactive Systems. There are a large number of electroactive systems (Table III) present in animal tissues, in plants, and in bacteria. Their function, however, is still unknown. Pyocyanine, for example, an electroactive system with large radical formation constant, would be an ideal catalyst for the respiration of *Pseudomonas aeruginosa.* Yet the respiration of these bacteria is entirely inhibited by hydrogen cyanide (Barron and Friedemann, 1941).

Sluggish Systems

The sluggish oxidation-reduction systems are characterized by their inertness toward molecular oxygen, and by their sluggishness in giving their proper e.m.f. values in the presence of noble metal electrodes. They possess the role of intermediary agents for the transfer of electrons when acting as components of oxidases. Besides cytochrome *c*, the most impor-

TABLE V

OXIDATION-REDUCTION POTENTIALS OF NONENZYMATIC SLUGGISH SYSTEMS

System	E_0' at pH 7.0
	volts
Cytochrome *c*	+0.255
Ascorbic acid	+0.060
Diphosphopyridine nucleotide	+0.282
Glutathione	−0.40

tant substances among this group are the pyridine nucleotides, glutathione, and ascorbic acid (Table V).

Pyridine Nucleotides. The pyridine nucleotides are reversible systems in which oxidation-reduction is performed by steps, that is, through radical formation and electron by electron. They are derivatives of nicotine amide. One of them, diphosphopyridine nucleotide (DPN), was detected in 1906 by Harden and Young as a coenzyme of alcoholic fermentation. The other, triphosphopyridine nucleotide (TPN), was discovered in 1932 by Warburg and Christian. The laboratory of Warburg deserves chief

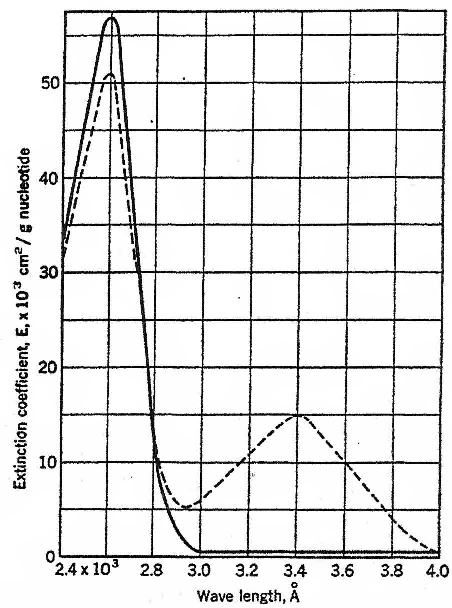


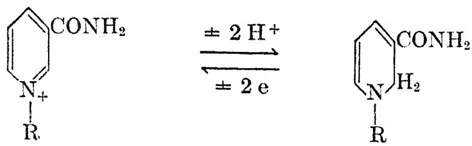
Fig. 3.—The absorption spectrum of diphosphopyridine nucleotide.

—, oxidized state; ---, reduced state

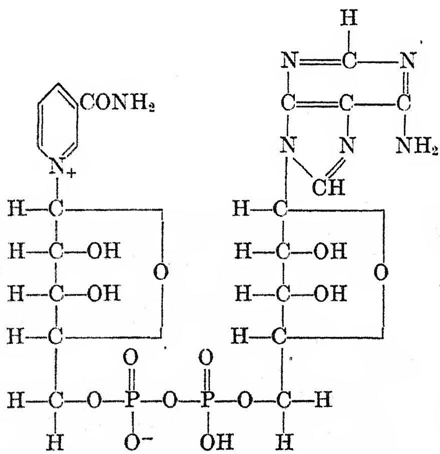
credit for the determination of the chemical constitution of these nicotine amide derivatives, although a great deal of work was also done at Euler's laboratory, where the properties of DPN were studied from 1921 to 1934. Both DPN and TPN are nicotinic amide–adenine dinucleotides. The transformation of one into the other is performed either enzymatically or by chemical phosphorylation. Triphosphopyridine nucleotide can be reduced with hydrosulfite and reoxidized by ferricyanide or 2,6-dichlorophenolindophenol.* The reduced compounds have an absorption band at 3400 Å (Fig. 3), which is widely used for their determination and for kinetic studies on the rate of reduction.

* Personal communication from Dr. E. Haas.

On catalytic hydrogenation with hydrogen and platinum black, phosphopyridine nucleotides take up six hydrogen atoms as a result of reduction of the adenine nucleus. In enzymatic oxidation-reduction, the nicotine amide nucleus is the center of electron transfer. The nicotine amide is bound as a quaternary pyridinium compound:



The oxidized compounds are stable in acid solutions but extremely labile in alkaline solutions, whereas the reduced compounds are stable in alkali and are sensitive toward acids. Alkaline as well as acid hydrolysis separates the carbohydrate from the nicotine amide. The structural formula of DPN seems as given below. The pentose of the nicotine amide part



Diphosphopyridine nucleotide

is identical with *d*-ribose (Schlenk, 1942; Euler *et al.*, 1942). In TPN it seems probable that the third phosphoric acid group is linked to the adenylic acid part of the molecule, perhaps in much the same way as in yeast adenylic acid.

DPN can be determined manometrically by the method of Jandorf, Klemperer, and Hastings (1941), which is based on the series of reactions postulated by Warburg and Christian to occur when a glycolyzing system (in this case acetone powder of cat muscle, deprived of DPN by charcoal treatment) is incubated in bicarbonate buffer and arsenate. Under the conditions described by the authors, the amount of carbon dioxide liberated

by the acid production is proportional to the amount of DPN. It can be determined spectrophotometrically more easily by measuring the reduction at 6100 Å of 2,6-dichlorophenolindophenol in the system containing activating protein of alcohol oxidase (as prepared by Negelein and Wulff, 1937), flavoprotein, alcohol, semicarbazide, DPN, and 2,6-dichlorophenolindophenol. In this system, $(DPN)H_2$ is reoxidized by the dye in the presence of catalytic amounts of flavoprotein, and the amount of reduced dye* is proportional to the DPN concentration. The growth-promoting properties of certain microorganisms, such as *Hemophilus influenzae* (Kohn, 1938) and *Hemophilus parainfluenzae* (Hoagland, Ward, Gilder, and Shank, 1942) can also be used for the determination of DPN.

TPN can be determined with hexose-6-phosphate as substrate by the use of hexosephosphate oxidase (activating protein, TPN, alloxazine–mononucleotide–protein, and cytochrome *c*). If TPN is made the rate-determining factor, its concentration can be measured by following the rate of cytochrome *c* reduction (Haas *et al.*, 1942).

The pyridine nucleotides combine reversibly with proteins:

$$\text{DPN} + \text{protein} \rightleftharpoons \text{protein–DPN}$$

$$(\text{DPN})\text{H}_2 + \text{protein} \rightleftharpoons \text{protein–(DPN)H}_2$$

The value of the dissociation constants of these complexes is known for only a few of them because of lack of purification of the proteins. In alcohol oxidase, the $K_{ox.}$ value for the first reaction is 9.5×10^{-5}, and the $K_{red.}$ value for the second reaction is 3.2×10^{-5} (Negelein and Wulff, 1937). As a consequence, the equilibrium is shifted toward acetaldehyde reduction. In hexosemonophosphate oxidase, the $K_{ox.}$ and $K_{red.}$ values for the reactions TPN + protein $\rightleftharpoons$ protein–TPN and $(TPN)H_2$ + protein $\rightleftharpoons$ protein–$(TPN)H_2$ are identical, 1.1×10^{-5} (Negelein and Haas, 1935; Negelein and Gerischer, 1936).

Since the dissociation constants of the reversible complexes DPN–protein and $(DPN)H_2$–protein have different values, the oxidation-reduction potentials of pyridine nucleotides will vary with the degree of association of pyridine and protein. The pyridine nucleotides are the oxidation-reduction systems which invariably combine with the activating protein (dehydrogenase), thus acting as the direct oxidizing agents of the oxidizable substrates. DPN acts as the catalyst for the fermentative and oxidative phase of carbohydrate metabolism and for the oxidation of certain fatty acids and amino acids. TPN acts as the catalyst for the oxidation of hexoses (glucose, glucose-6-phosphate), isocitric acid, and sometimes amino

* The use of dye to measure DPN concentration was suggested to the author by Dr. E. Haas.

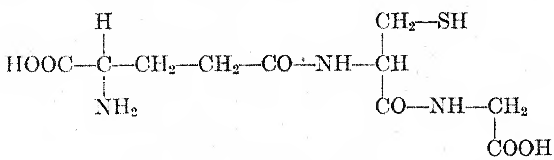
acids. The oxidation of the reduced compounds seems to be performed as a rule by flavoproteins, for example, alloxazine mononucleotides for the oxidation of $(TPN)H_2$, and alloxazine dinucleotides for the oxidation of $(DPN)H_2$. There is, in Table VI, a list of oxidation-reduction reactions in which pyridine nucleotides act as components of enzyme systems. As a rule, reactions catalyzed by DPN are easily reversible oxidation-reduction systems, while reactions catalyzed by TPN are not reversible.

TABLE VI

THE PYRIDINE NUCLEOTIDES AS COMPONENTS OF ENZYME SYSTEMS

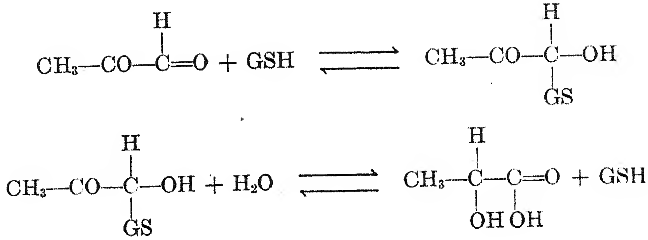
Property of protein	Pyridine nucleotide	Reductant	Oxidant
Activating	TPN	Hexose monophosphate	Flavoprotein
Activating	TPN	Phosphohexonate	Flavoprotein
Activating	TPN	Isocitrate	Flavoprotein
Activating	TPN	Glucose	Flavoprotein
Activating	TPN	Glutamate	Flavoprotein
Activating	DPN	Diphosphoglyceraldehyde	Pyruvate through flavoprotein (?)
Activating	DPN	Alcohol	Flavoprotein (?)
Activating	DPN	Malate	Flavoprotein (?)
Activating	DPN	Aldehyde	Flavoprotein (?)
Activating	DPN	Glycerophosphate	Flavoprotein (?)
Activating	DPN	β-Hydroxy butyrate	Flavoprotein (?)
Activating	DPN	Formate	Flavoprotein (?)
Activating	DPN	Lactate	Flavoprotein (?)
Activating	DPN	Diphosphoglyceraldehyde	Oxalacetate through flavoprotein (?)

Glutathione. Another widely distributed sluggish system is glutathione. It was discovered by Hopkins in 1921, and its constitution is now known to be the tripeptide glutamyl-cysteinyl-glycine (GSH).



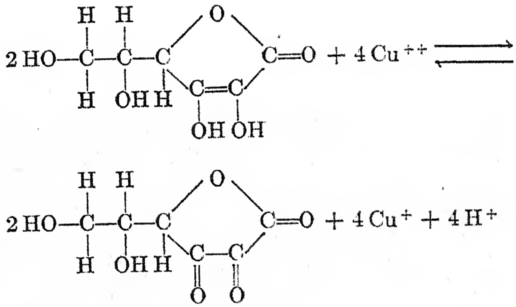
Glutathione

GSH is not oxidized by molecular oxygen. The oxidation proceeds rapidly in the presence of catalysts like copper and hemochromogens (Lyman and Barron, 1937). It is widely distributed in animal tissues and yeast, and is present in some plant tissues. So far, glyoxalase is the only enzyme system in which glutathione acts as a component of the system; glutathione combines with methylglyoxal, and the dissociation of the compound is accelerated by sodium bicarbonate in the presence of the enzyme:



The most important role of glutathione seems to be that of reactivating the protein enzymes possessing sulfhydryl groups essential for enzymatic activity. Whenever these groups are oxidized during the continuous process of oxidation-reductions going on in the living cells, glutathione will reduce the inactive —S—S— group back to the active —SH form. In plant tissues, this role of glutathione may be taken by ascorbic acid.

Ascorbic Acid. In his search for catalysts of biological oxidations, Szent-Györgyi (1928) discovered ascorbic acid. As with all sluggish systems, ascorbic acid is not oxidized by molecular oxygen unless a catalyst is present. The most powerful oxidation catalyst is the cupric ion (Barron *et al.*, 1936), and the reaction is as follows:



$$4\,Cu^{+} + 4\,H^{+} + 2\,O_2 \longrightarrow 4\,Cu^{++} + 2\,H_2O_2$$

$$2\,H_2O_2 \longrightarrow 2\,H_2O + O_2$$

The oxidation of ascorbic acid is reversible at values below *p*H 6. Above *p*H 6 the oxidized compound is destroyed, the rate of destruction increasing as the *p*H value increases. In 1931, Szent-Györgyi found that ascorbic acid was rapidly oxidized by cabbage leaves. Later it was found that this oxidation is performed by ascorbic acid oxidase—a copper–protein enzyme.

Although ascorbic acid is abundant in many plants, its role in biological oxidations is not yet known. Some plant tissues (cabbage, squash, watercress) contain ascorbic acid oxidase, others do not. Szent-Györgyi (1937) and Huszák (1937) think that peroxides, peroxidases, and ascorbic

acid play an important part in plant respiration. Molecular oxygen interacts with ascorbic acid oxidase and oxidizes ascorbic acid. Oxygen is thus reduced to hydrogen peroxide. The hydrogen peroxide formed reacts with peroxidase and oxidizes a flavone (or flavonol or flavanone). The oxidized flavone in its turn oxidizes a second molecule of ascorbic acid. Dehydroascorbic acid is again reduced by the tissues. It is contended that such reduction is performed by glutathione or on the oxidation of citric acid. There is as yet no proof of the correctness of these contentions.

As has already been stated, these sluggish systems, although thermodynamically reversible, do not give the calculated e.m.f. values when noble metal electrodes are in contact with different ratios of oxidant and reductant. As a result, widely discrepant values of the oxidation-reduction potentials are seen in the literature. The oxidation-reduction potentials of the systems given in Table V were obtained with the use of electroactive mediators.

Enzymatic-Sluggish Systems

When an indifferent electrode, gold or bright platinum, is immersed in an oxygen-free buffered solution containing a mixture of lactate and its oxidation product, pyruvate, no potential indicative of electron transfer is obtained at the electrode. The addition of the enzyme hydroxyoxidase from gonococci gives to this system, lactate–pyruvate, a slight tendency to transfer electrons to the electrode; that is, an erratic potential is obtained which drifts slowly toward negative values without attainment of equilibrium. When an electroactive system (for example, a dye) previously shown to be only partially reduced by this system is added, a stable, reproducible potential is obtained at the end of one to two hours. Although the potential is due directly to an electron transfer from the electroactive system, this system acts simply as a mediator, for its reduction has been brought about through equilibrium with the system lactate–enzyme–pyruvate. The values obtained may therefore be taken as representing the potential of this system (Barron and Hastings, 1934). The potentials of these reversible systems thus require the mediation of two factors: the enzyme, which acts as a perfect catalyst; and the electroactive system, which is used to measure the potential of the system. These potentials may therefore be measured either electrometrically or colorimetrically.

Quastel and Whetham (1924) discovered the existence of the first of these systems, succinate–fumarate. The reason for this early discovery can be traced to the wide use of methylene blue as an indicator of oxidation. It happens that the potential of the succinate–fumarate system lies around that of methylene blue. Since 1924 a number of enzymatic-sluggish oxidation-reduction systems have been discovered covering a wide range of po-

tentials, from the hydrogen potential up to the methylene blue potential (Table VII).

TABLE VII

OXIDATION-REDUCTION POTENTIALS OF ENZYMATIC-SLUGGISH SYSTEMS

System	E_0' at pH 7.0
	volts
Succinate ⇌ fumarate	0.0
Glutamate ⇌ α-keto glutarate	−0.030
d(−)Alanine ⇌ ammonium pyruvate	−0.048
α-Hydroxy glutarate ⇌ α-keto glutarate	−0.07
Malate ⇌ oxalacetate	−0.102
Ethyl alcohol ⇌ acetaldehyde	−0.165
Lactate ⇌ pyruvate	−0.180
Isopropyl alcohol ⇌ acetone	−0.251
β-Hydroxy butyrate ⇌ acetoacetate	−0.293
Xanthine ⇌ uric acid	−0.361
Hypoxanthine ⇌ xanthine	−0.371
Formate ⇌ hydrogen + carbon dioxide	−0.420
Hexose phosphate ⇌ phosphohexonic acid	−0.43
Glucose ⇌ gluconic acid	−0.45

It must be pointed out that the efficiency of these reversible systems is not high. For example, for the reaction succinate–fumarate, the ratio $-\Delta F/-\Delta H$ is 0.68; for the reaction lactate–pyruvate, it is 0.53. In other words, the theoretical maximum work derivable from these reactions is approximately half the total heat change.

It is apparent that these systems might take part in biological oxidations by acting as reversible steps in the transfer of electrons from foodstuff to molecular oxygen. Borsook (1935) gave some examples of this thermodynamic possibility by oxidizing sluggish-enzymatic systems of negative potential by systems of more positive potential through the mediation of suitable electroactive systems. The extent of oxidation of the sluggish system A by the sluggish system B is determined by the equation below, in which the intermediary catalyst and the dissociation constants have been omitted for the sake of simplicity:

$$E'_{0A} - E'_{0B} = \frac{RT}{nF} \ln \frac{(A_{\text{ox.}})(B_{\text{red.}})}{(A_{\text{red.}})(B_{\text{ox.}})}$$

Flavoproteins, the reversibleoxidation-reduction pigments, and iron-porphyrins may act as the electron mediators between sluggish-enzymatic systems. Szent-Györgyi's "cycle" or Krebs's "cycles" are only applications of these concepts. Szent-Györgyi's hypothesis that succinate–fumarate and malate–oxaloacetate may act as auxiliary catalysts by interposing these reversible steps in the series of oxidation-reductions has solid thermodynamic foundation. In fact, both systems have potentials suitable for carbohydrate oxidation. A number of papers have appeared maintaining

that plant respiration requires these auxiliary catalysts. Unfortunately, the assertion is based only on the fact that succinate and citrate are found in plants. Although not a known fact, it may very well be that reversible systems act in plants as accessory catalysts by coupled oxidation-reductions between split products of foodstuff through the mediation of suitable electroactive mediators. Among those systems attention must be paid to the naphthoquinone derivatives present in plants and identified as vitamin K.

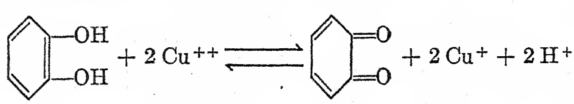
TYPES OF OXIDIZING ENZYME SYSTEMS

It is now established that oxidation enzyme systems are made up of two main components: a specific protein, the activating protein or dehydrogenase; and a number of reversible oxidation-reduction systems which transfer electrons from oxidizable substrate to molecular oxygen. It is also established that if there is only one reversible system it is electroactive and autoxidizable, combining readily with molecular oxygen; if there are two systems one is sluggish and nonautoxidizable, and the other electroactive. If there are more, the series of oxidation-reduction systems is made up as follows: sluggish system $\rightleftharpoons$ electroactive system $\rightleftharpoons$ sluggish system $\rightleftharpoons$ electroactive system $\rightleftharpoons$ oxygen.

First Type: Metalloproteins

The first type is the simplest of all oxidases, since it is made up of a metal ion that can be reversibly oxidized and reduced and a specific protein. The enzyme is a metalloprotein.

Polyphenol oxidase is a typical example. The enzyme is a copper–protein (Kubowitz, 1938, 1938a). Potato polyphenol oxidase oxidizes *o*-diphenols such as catechol, adrenaline, dihydroxyphenylalanine, etc. Monophenols such as phenol and *p*-cresol are oxidized, but not immediately. The oxidation is effected as follows:



$$2\,Cu^{+} + O_2 \longrightarrow 2\,Cu^{++} + 2\,O^{-}$$

$$2\,O^{-} + 2\,H^{+} \longrightarrow H_2O_2 \longrightarrow H_2O + {}^1/_2\,O_2$$

The first reaction probably occurs in two steps via formation of a semiquinone. In other words, one molecule of copper–protein oxidizes catechol to the semiquinone, and another molecule oxidizes this to the quinone form. The enzyme contains about 0.2 per cent of copper. This copper can be separated from the protein by dialysis in the presence of hydrogen cyanide. Iron, cobalt, nickel, manganese, and zinc are inactive as prosthetic groups.

Tyrosinase is another copper–protein (Dalton and Nelson, 1939). It is found in plants, molds, crustacea, and mollusks. Potatoes, dahlia bulbs, wheat bran, and certain mushrooms are good vegetable sources. Tyrosinase acts upon adrenaline, tyrosine, phenol, catechol, *m*-cresol, *p*-cresol, homocatechol, pyrogallol, and dopa (3,4-dihydroxyphenylalanine). It does not attack *o*-cresol, *p*-phenylenediamine, resorcinol, hydroquinone, or ascorbic acid.

Laccase, the copper–protein (Keilin and Mann, 1939, 1940) found in the latex of the lac tree, oxidizes phenols to *o*- and *p*-quinones. Laccase oxidizes guaiacol, catechol, hydroquinone, pyrogallol, *p*-phenylenediamine, and various phenols, but does not oxidize tyrosine, resorcinol, or *p*-cresol.

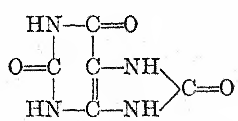
Ascorbic acid oxidase is also a copper–protein enzyme (Tadokoro and Takasugi, 1939). The oxidation proceeds in the same manner as the oxidation by ionic copper already discussed. This enzyme seems to be present in only a few plant tissues.

In general, the copper–protein enzymes have an optimum activity from *p*H 6 to 8. The enzymes are inhibited by heavy metal inhibitors such as cyanide and copper-combining substances such as diethyldithiocarbamate. The contention that no hydrogen peroxide is produced in these oxidations is contrary to the accepted mechanism of oxidation with copper as oxidation catalyst. The hydrogen peroxide formed must be destroyed as soon as it is produced, so that its detection is difficult.

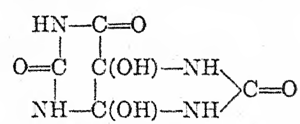
Uricase converts uric acid (I) to allantoin. The reaction proceeds according to the equation:

$$\text{Uric acid} + 2\,H_2O + O_2 \longrightarrow \text{allantoin} + CO_2 + H_2O_2$$

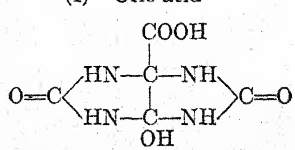
The first state in the oxidation may consist in the formation of 4,5-glycoluric acid (II). This rearranges to form oxyacetylenediureine carboxylic acid (III), which spontaneously gives rise to allantoin (IV) by decarboxylation. However, the mechanism of this oxidation has not been established.



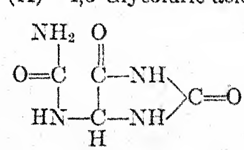
(I) Uric acid



(II) 4,5-Glycoluric acid



(III) Oxyacetylenediureinecarboxylic acid



(IV) Allantoin

The optimum hydrogen-ion concentration for uricase action is *p*H 8.8. It is reversibly inactivated by cyanide. Davidson (1942) has purified the

enzyme to a certain extent and reports that the protein contains iron and zinc in large amounts.

The list of metalloproteins acting as oxidases has not yet been exhausted. Certainly, as purification procedures improve, many of those "unclassified enzymes" (monoamine oxidase, diamine oxidase, glucose oxidase, lipoxidase) may be found to possess a heavy metal or flavoprotein as the prosthetic group directly connected with the oxidation-reduction process.

Second Type: Flavoprotein Enzymes

The only difference between metalloproteins and flavoprotein enzymes is that, in the latter, the prosthetic group responsible for the oxidation of the substrate is an alloxazine instead of a heavy metal. Reduced alloxazine is reoxidized by atmospheric oxygen with formation of hydrogen peroxide. This accumulates because flavins—in contradistinction to heavy metals—do not split the peroxide. *d*-Amino acid oxidases, xanthine oxidase, liver aldehyde oxidase, and pyruvate oxidase in *Lactobacillus delbrueckii,* belong to this group.

Third Type: Flavoprotein–Cytochrome Enzymes

The complexity of some oxidases starts with this group. The substrate, activated by the activating protein (dehydrogenase), is oxidized by flavin; reduced flavin is oxidized by cytochrome; cytochrome is oxidized by cytochrome oxidase; finally, cytochrome oxidase is oxidized by atmospheric oxygen. In other words, the amount of energy being released on oxidation of substrate by molecular oxygen is delivered in at least four steps, with an enormous increase of the free useful energy obtained in the oxidation process:

$$\text{Substrate} + \text{flavin} \rightleftharpoons \text{oxidized substrate} + \text{flavin}\cdot H_2$$

$$\text{Flavin}\cdot H_2 + 2\,Fe_{cyt.}^{+++} \rightleftharpoons 2\,Fe_{cyt.}^{++} + \text{flavin} + 2\,H^+$$

$$2\,Fe_{cyt.ox.}^{+++} + 2\,Fe_{cyt.}^{++} \rightleftharpoons 2\,Fe_{cyt.}^{+++} + 2\,Fe_{cyt.ox.}^{++}$$

$$2\,Fe_{cyt.ox.}^{++} + O_2 \rightleftharpoons 2\,Fe_{cyt.ox.}^{+++} + 2\,O^-$$

$$2\,H^+ + 2\,O^- \longrightarrow H_2O_2 \longrightarrow H_2O + {}^1/_2\,O_2$$

As pointed out previously, although the first two equations seem to involve two-electron oxidation-reductions, in reality these oxidations must be performed step by step through intermediate formation of a semiquinone or free radical. The same considerations apply to all the apparent two-electron oxidations.

Oxidases belonging to this group have not yet been broken down into their purified components. However, typical examples are α-hydroxyoxidase and α-ketoxidase from gonococci. Lactate is oxidized to pyruvate by the first enzyme; and pyruvate is oxidized to acetate and carbon dioxide by the second enzyme. In both cases oxidation is produced by the oxidation-reduction systems: flavoprotein and cytochromes. Succinoxidase, choline oxidase, and ketoglutarate oxidase seem to belong to this type.

Fourth Type: Pyridine–Protein–Flavoprotein Enzymes

In this group of enzymes the sluggish system pyridine–nucleotide is introduced either as the diphospho or the triphospho compound. The activated substrate in these oxidases is oxidized by DPN or by TPN; the reduced pyridine nucleotides are oxidized by flavoproteins; the reduced flavoproteins by molecular oxygen:

$$\text{Substrate} + \text{TPN} \rightleftharpoons \text{oxidized substrate} + (\text{TPN})\text{H}_2$$

$$(\text{TPN})\text{H}_2 + \text{flavoprotein} \rightleftharpoons \text{TPN} + \text{flavoprotein}\cdot\text{H}_2$$

$$\text{Flavoprotein}\cdot\text{H}_2 + \text{O}_2 \rightleftharpoons \text{flavoprotein} + \text{H}_2\text{O}_2$$

The oxidation of hexose monophosphate in the presence of Warburg's *Zwischenferment* (activating protein), TPN, and Warburg's old "yellow ferment" could be presented as an example of this type of oxidase (Warburg and Christian, 1932, 1933, 1933a). Such oxidases must exist in cells lacking the cytochrome system. Lactate oxidase and glycerol oxidase prepared from cytochrome-lacking bacteria are oxidases in which lactate and glycerol are oxidized by DPN and flavoproteins.

Fifth Type: Pyridine–Protein–Flavoprotein-Cytochrome System Enzymes

In the fifth type of oxidases, electron transfer from substrate to molecular oxygen is effected through the mediation of two sluggish systems (pyridine nucleotide and cytochrome) and two electroactive systems (flavoprotein and cytochrome oxidase):

$$\text{Substrate} + \text{TPN} \rightleftharpoons \text{oxidized substrate} + (\text{TPN})\text{H}_2$$

$$(\text{TPN})\text{H}_2 + \text{flavoprotein} \rightleftharpoons \text{TPN} + \text{flavoprotein}\cdot\text{H}_2$$

$$\text{Flavoprotein}\cdot\text{H}_2 + 2\,\text{Fe}_{\text{cyt.}}^{+++} \rightleftharpoons \text{flavoprotein} + 2\,\text{Fe}_{\text{cyt.}}^{++} + 2\,\text{H}^+$$

$$2\,\text{Fe}_{\text{cyt.}}^{++} + 2\,\text{Fe}_{\text{cyt.ox.}}^{+++} \rightleftharpoons 2\,\text{Fe}_{\text{cyt.}}^{+++} + 2\,\text{Fe}_{\text{cyt.ox.}}^{++}$$

$$2\,\text{Fe}_{\text{cyt.ox.}}^{++} + \text{O}_2 \rightleftharpoons 2\,\text{Fe}_{\text{cyt.ox.}}^{++} + 2\,\text{O}^-$$

$$2\,\text{O}^- + 2\,\text{H}^+ \longrightarrow \text{H}_2\text{O}_2 \longrightarrow \text{H}_2\text{O} + {}^1/_2\,\text{O}_2$$

The hexosemonophosphate oxidase, as presented by Haas *et al.* (1940), is the most representative of this type of oxidase. This enzyme system differs from that of Warburg and Christian in that the old "yellow enzyme" has been replaced by a flavoprotein which reduces cytochrome *c* at a speed many times higher than the speed of reduction of molecular oxygen. It is plausible to assume that the large number of enzymes present in cytochrome containing cells with pyridine–proteins as the first oxidation system belong to this group (see Table VI).

Coupled Oxidation-Reductions

In isolated systems, electron transfer from oxidizable substrate to molecular oxygen is performed through the mediation of the different reversible systems already discussed. However, when a number of these systems are present together (as happens normally in living cells) the continuity of electron transport schematized in the different types of oxidations possibly does not exist. Instead, electron transfer might proceed through coupled oxidation-reductions. Szent-Györgyi (1937a) gives an interesting example. In the presence of the respective activating proteins, DPN and flavoproteins:

$$\text{Malate} + \text{fumarate} \rightleftharpoons \text{oxalacetate} + \text{succinate}$$

Electron transfer in this coupled oxidation-reduction probably occurs through flavoproteins. Another possible coupled oxidation-reduction is the following:

$$\text{Pyruvate} + \text{fumarate} \rightleftharpoons \text{acetate} + \text{succinate}$$

$$\text{Pyruvate} + \text{flavoprotein} \rightleftharpoons \text{acetyl phosphate} + \text{flavoprotein}\cdot H_2$$

$$\text{Fumarate} + \text{flavoprotein}\cdot H_2 \rightleftharpoons \text{succinate} + \text{flavoprotein}$$

The last two reactions have been found to exist. Electron transfer from one system to another through flavoprotein would insure continuity of the oxidation process and would explain the catalytic effect that fumarate has on cellular respiration. Systems similar to these have been presented by Krebs (1943) in the series of "cycles" that he has offered to explain the oxidation of pyruvate.

Phosphorylations and Oxidation-Reductions

In the description of the components of oxidation enzyme systems, phosphorus has rarely been mentioned. Yet there are a number of enzyme reactions, particularly in carbohydrate metabolism, which do not proceed in the absence of phosphorus. The participation of phosphorus in carbohy-

drate oxidation has been demonstrated by Cori (1942) and by Ochoa (1941). The oxidation of pyruvate seems connected with phosphorylation of adenylic acid to adenosine triphosphate, which transfers its labile phosphate groups either to hexose monophosphate or to glucose. This linkage between oxidation and phosphate turnover has now been thoroughly established (see Lipmann's discussion, 1941), the oxidation process transferring energy to labile phosphate, and the labile phosphate energy being used directly for cellular activity.

BIBLIOGRAPHY

AGNER, K. (1941). Verdoperoxidase. *Acta Physiol. Scand.* 2, Suppl. VIII:5–62.

ALTSCHUL, A. M., and HOGNESS, T. R. (1938). Spectroscopic evidence for the existence of carboxycytochrome *c*. *J. Biol. Chem.* 124:25–31.

ANSON, M. L., and MIRSKY, A. E. (1925). On haemochromogen and the relation of protein to the properties of the haemoglobin molecule. *J. Physiol.* 60:50–68.

BALL, E. G. (1934). Studies of oxidation-reduction. XXI. Phthiocol the pigment of the human tubercle bacillus. *J. Biol. Chem.* 106:515–524.

—— (1936). Studies on oxidation-reduction. XXII. Lapachol, lomatiol, and related compounds. *J. Biol. Chem.* 114:649–655.

—— (1938). Über die Oxydation und Reduktion der drei Cytochrom-Komponenten. *Biochem. Z.* 295:262–264.

—— (1939). Xanthine oxidase: purification and properties. *J. Biol. Chem.* 128:51–67.

—— (1939a). The role of flavoproteins in biological oxidations. *Cold Spring Harbor Symposia Quant. Biol.* 7:100–110.

——, CHEN, T.-T., and CLARK, W. M. (1933). Studies on oxidation-reduction. XX. Epinephrine and related compounds. *J. Biol. Chem.* 102:691–719.

BANGA, I., and PHILPOT, E. (1939). Über die Dioxymaleinsäureoxydase. *Z. physiol. Chem.* 258:147–159.

——, ——, and SZENT-GYÖRGYI, A. (1938). Dioxymaleic acid oxidase. *Nature* 142:874.

——, SZENT-GYÖRGYI, A., and VARGHA, L. (1932). Über das Co-Ferment der Milchsäureoxydation. *Z. physiol. Chem.* 210:228–235.

BARRON, E. S. G. (1932). The rate of autoxidation of oxidation-reduction systems and its relation to their free energy. *J. Biol. Chem.* 97:287–302.

—— (1937). Studies on biological oxidations. IX. The oxidation-reduction potentials of blood hemin and its hemochromogens. *J. Biol. Chem.* 121:285–312.

——, DE MEIO, R. H., and KLEMPERER, F. (1936). Studies on biological oxidations. V. Copper and hemochromogens as catalysts for the oxidation of ascorbic acid. The mechanism of the oxidation. *J. Biol. Chem.* 112:625–640.

—— and FRIEDEMANN, T. E. (1941). Studies on biological oxidations. XIV. Oxidation by microorganisms which do not ferment glucose. *J. Biol. Chem.* 137:593–610.

——, GOLDINGER, J. M., LIPTON, M. A., and LYMAN, C. M. (1941). Studies on biological oxidations. XVII. The effect of thiamine on the metabolism of α-ketoglutarate. *J. Biol. Chem.* 141:975–979.

—— and HASTINGS, A. B. (1933). Studies on biological oxidations. II. The oxidation of lactic acid by α-hydroxyoxidase, and its mechanism. *J. Biol. Chem.* 100:155–182.

—— and —— (1934). Studies on biological oxidations. III. The oxidation-reduction potential of the system lactate–enzyme–pyruvate. *J. Biol. Chem.* 107:567–578.

—— and JACOBS, H. R. (1938). Oxidations produced by hemolytic streptococci. *J. Bact.* 36:433–449.

PARRON, E. S. G., and LYMAN, C. M. (1938). Studies on biological oxidations. XI. The metabolism of pyruvic acid by animal tissues and bacteria. *J. Biol. Chem.* 127:143–161.

—— and —— (1941). Studies on biological oxidations. XV. The rates of reduction of thiamine and diphosphothiamine. *J. Biol. Chem.* 141:951–956.

——, ——, LIPTON, M. A., and GOLDINGER, J. M. (1941). Studies on biological oxidations. XVI. The effect of thiamine on condensation reactions of pyruvate. *J. Biol. Chem.* 141:957–973.

BORSOOK, H. (1935). Reversible and reversed enzymatic reactions. *Ergeb. Enzymforsch.* 4:1–41.

CANNAN, R. K. (1926). Electrode potentials of hermidin, the chromogen of *Mercurialis perennis*. *Biochem. J.* 20:927–937.

—— (1927). Echinochrome. *Biochem. J.* 21:184–189.

CLARK, W. M., and COHEN, B. (1923). Studies on oxidation-reduction. I. Introduction. *U. S. Pub. Health Repts.* 38:443–461.

—— and —— (1923a). Studies on oxidation-reduction. II. An analysis of the theoretical relations between reduction potentials and *p*H. *U. S. Pub. Health Repts.* 38:666–683.

—— and PERKINS, M. E. (1940). Metalloporphyrins. V. Spectrophotometric study of pyridine coproporphyrin I. *J. Biol. Chem.* 135:643–657.

——, TAYLOR, J. F., DAVIES, T. H., and VESTLING, C. S. (1940). Metalloporphyrins. I. Coordination with nitrogenous bases. Theoretical relations. *J. Biol. Chem.* 135:543–567.

CONANT, J. B., ALLES, G. A., and TONGBERG, C. O. (1928). The electrometric titration of hemin and hematin. *J. Biol. Chem.* 79:89–93.

—— and TONGBERG, C. O. (1930). The oxidation-reduction potentials of hemin and related substances. I. The potentials of various hemins and hematins in the absence and presence of pyridine. *J. Biol. Chem.* 86:733–741.

CORI, C. F. (1942). Phosphorylation of carbohydrates. In *A Symposium on Respiratory Enzymes*, pp. 175–189. Univ. Wisconsin Press, Madison.

CORRAN, H. S., and GREEN, D. E. (1938). The isolation of a flavoprotein from cow's milk. *Biochem. J.* 32:2231–2240.

DALTON, H. R., and NELSON, J. M. (1939). Tyrosinase from the wild mushroom, *Lactarius piperatus*. *J. Am. Chem. Soc.* 61:2946–2950.

DAVIDSON, J. N. (1942). The purification of uricase. II. Some properties of purified uricase. *Biochem. J.* 36:252–258.

DEWAN, J. G., and GREEN, D. E. (1937). Coenzyme-linked reactions between dehydrogenase systems. *Biochem. J.* 31:1074–1085.

DIXON, M. (1937). Action of iodoacetate on dehydrogenases and alcoholic fermentation. *Nature* 140:806.

ELEMA, B. (1931). Studies on the oxidation-reduction of pyocyanine. Part II. Redox potentials of pyrocyanine. *Rec. trav. chim.* 50:807–826.

—— (1933). Oxidation-reduction potentials of chlororaphine. *Rec. trav. chim.* 52:569–583.

EULER, H. v., and HELLSTRÖM, H. (1938). Zur Kenntnis der enzymatischen Wasserstoffüberträger im Muskel. *Z. physiol. Chem.* 252:31–40.

——, KARRER, P., and USTERI, E. (1942). Die Zucker der Cozymase. *Helv. Chim. Acta* 25:323–325.

EYRING, H. (1935). The activated complex in chemical reactions. *J. Chem. Phys.* 3:107–116.

FIESER, L. F., and FIESER, M. (1935). A new diene synthesis of anthraquinones. *J. Am. Chem. Soc.* 57:1679–1681.

FILITTI, S. (1935). Sur l'équilibre d'oxydoréduction des oxypurines. *J. chim. phys.* 32:1–45.

FISCHER, F. G., and EYSENBACH, H. (1937). Eine neuartige enzymatische Hydrierung der Fumarsäure. Biochemische Hydrierung. VI. *Ann.* 530: 99–120.

FISCHER, F. G., ROEDIG, A., and RAUCH, K. (1939). Fumarathydrase, ein gelbes Ferment. *Naturwissenschaften* **27**:197.

FISHBERG, E. H., and DOLIN, B. T. (1933). The biological action of strongly positive oxidation-reduction systems. *J. Biol. Chem.* **101**:159–171.

FRIEDHEIM, E. A. H. (1932). La fonction respiratoire du pigment du *Bacillus violaceus*. *Compt. rend. soc. biol.* **110**:353–356.

—— (1933). Sur la fonction respiratoire du pigment rouge du *Penicillium phoeniceum*. *Compt. rend. soc. biol.* **112**:1030–1032.

—— (1933a). Das Pigment von *Halla Parthenopea*, ein accessorischer Atmungs-Katalysator. *Biochem. Z.* **259**:257–268.

—— (1934). Natural reversible oxidation-reduction systems as accessory catalysts in respiration: juglone and lawsone. *Biochem. J.* **28**:180–188.

FRUTON, J. S., and CLARKE, H. T. (1934). Chemical reactivity of cystine and its derivatives. *J. Biol. Chem.* **106**:667–691.

GORDON, A. H., GREEN, D. E., and SUBRAHMANYAN, V. (1940). Liver aldehyde oxidase. *Biochem. J.* **34**:764–774.

GREEN, D. E. (1934). Studies on reversible dehydrogenase systems. II. The reversibility of the xanthine-oxidase system. *Biochem. J.* **28**:1550–1560.

——, HERBERT, D., and SUBRAHMANYAN, V. (1940). On the isolation and properties of carboxylase. *J. Biol. Chem.* **135**:795–796.

——, WESTERFELD, W. W., VENNESLAND, B., and KNOX, W. E. (1941). Pyruvic and α-ketoglutaric carboxylases of animal tissues. *J. Biol. Chem.* **140**:683–684.

GYÖRGY, P., KUHN, R., and WAGNER-JAUREGG, T. (1936). Flavine und Flavoproteine als Vitamin B_2. *Z. physiol. Chem.* **223**:241–244.

HAAS, E. (1934). Cytochrom. *Naturwissenschaften* **22**:207.

—— (1938). Isolierung eines neuen gelben Ferments. *Biochem. Z.* **298**:378–390.

—— (1943). Cytochrome oxidase. *J. Biol. Chem.* **148**:481–493.

—— (1944). Separation of cytochrome oxidase into two components. *J. Biol. Chem.* **152**:695–696.

——, HARRER, C. J., and HOGNESS, T. R. (1942). Microdetermination of triphosphopyridine nucleotide. *J. Biol. Chem.* **142**:835–837.

——, HORECKER, B. L., and HOGNESS, T. R. (1939). The enzyme reduction of cytochrome *c;* a new enzyme. *J. Biol. Chem.* **130**:425–426.

——, ——, and —— (1940). The enzymic reduction of cytochrome *c*. Cytochrome *c* reductase. *J. Biol. Chem.* **136**:747–774.

HARDEN, A., and YOUNG, W. J. (1906). The alcoholic ferment of yeast-juice. *Proc. Roy. Soc. London* **B77**:405–420.

HILL, E. S. (1936). The two-step oxidation-reduction of phthiocol. *Proc. Soc. Exptl. Biol. Med.* **35**:363.

—— (1938). Two-step oxidation-reduction of lapachol, lomatiol and related compounds. *J. Am. Chem. Soc.* **60**:1990–1994.

HOAGLAND, C. L., WARD, S. M., GILDER, H., and SHANK, R. E. (1942). Studies on the nutrition of *Hemophilus influenzae*. I. The relationship between the utilization of coenzyme and hemin and the reduction of nitrate. *J. Exptl. Med.* **76**:241–252.

HOPKINS, F. G. (1921). An autoxidizable constituent of the cell. *Biochem. J.* **15**: 286–305.

——, MORGAN, E. J., and LUTWAK-MANN, C. (1938). The influence of thiol groups in the activity of dehydrogenases. II. (With an addendum on the location of dehydrogenases in muscle.) *Biochem. J.* **32**:1829–1848.

HOROWITZ, N. H., and BAUMBERGER, J. P. (1941). Studies on the respiratory pigment of *Urechis* eggs. *J. Biol. Chem.* **141**:407–415.

HUSZÁK, ST. (1937). Über die Funktion des Peroxydase-Systems der Pflanzen. *Z. physiol. Chem.* **247**:239–247.

JANDORF, B. J., KLEMPERER, F. W., and HASTINGS, A. B. (1941). A manometric method for the determination of diphosphopyridine nucleotide. *J. Biol. Chem.* **138**:311–320.

JOHNSON, F. H., and VAN SCHOUWENBURG, K. L. (1939). Decomposition of hydrogen peroxide by catalase. *Nature* **144**:634–635.

KARRER, P., BECKER, B., BENZ, F., FREI, P., SALOMON, H., and SCHÖPP, K. (1935). Zur Synthese des Lactoflavins. *Helv. Chim. Acta* **18**:1435–1448.

KEILIN, D. (1925). On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants. *Proc. Roy. Soc. London* **B98**:312–339.

—— and HARTREE, E. F. (1937). Preparation of pure cytochrome *c* from heart muscle and some of its properties. *Proc. Roy. Soc. London* **B122**:298–308.

—— and —— (1938). On the mechanism of the decomposition of hydrogen peroxide by catalase. *Proc. Roy. Soc. London* **B124**:397–405.

—— and —— (1940). Succinic dehydrogenase–cytochrome system of cells. Intracellular respiratory system catalysing aerobic oxidation of succinic acid. *Proc. Roy. Soc. London* **B129**:277–306.

—— and MANN, T. (1939). Laccase, a blue copper-protein oxidase from the latex of *Rhus succedanea*. *Nature* **143**:23–24.

—— and —— (1940). Some properties of laccase from the latex of lacquer trees. *Nature* **145**:304.

KOHN, H. I. (1938). The concentration of coenzyme-like substance in blood following the administration of nicotinic acid to normal individuals and pellagrins. *Biochem. J.* **32**:2075–2083.

KRAMPITZ, L. O., and WERKMAN, C. H. (1941). The enzymic decarboxylation of oxaloacetate. *Biochem. J.* **35**:595–602.

KREBS, H. A. (1943). The intermediary stages in the biological oxidation of carbohydrate. In *Advances in Enzymology*, Vol. III, pp. 191–252. Interscience, New York.

KUBOWITZ, F. (1938). Re-Synthese der Phenoloxydase aus Protein und Kupfer. *Biochem. Z.* **296**:443.

—— (1938a). Spaltung und Resynthese der Polyphenoloxydase und des Hämocyanins. *Biochem. Z.* **299**:32–57.

—— and LÜTTGENS, W. (1941). Zusammensetzung, Spaltung und Resynthese der Carboxylase. *Biochem. Z.* **307**:170–172.

KUHN, R., and BOULANGER, P. (1936). Beziehungen zwischen Reduktions-Oxydations-Potential und chemischer Konstitution der Flavine. *Ber.* **69**:1557–1566.

——, REINEMUND, K., WEYGAND, F., and STRÖBELE, R. (1935). Über die Synthese des Lactoflavins (Vitamin B_2). *Ber.* **68**:1765–1774.

—— and RUDY, H. (1935). Synthetische Vitamin-B_2-Phosphorsäure. *Ber.* **68**:383–386.

LA MER, V. K. (1933). Chemical kinetics. The temperature dependence of the energy of activation. The entropy and free energy of activation. *J. Chem. Phys.* **1**:289–296.

LIPMANN, F. (1939). An analysis of the pyruvic acid oxidation system. *Cold Spring Harbor Symposia Quant. Biol.* **7**:248–259.

—— (1941). Metabolic generation and utilization of phosphate bond energy. In *Advances in Enzymology*, Vol. I, pp. 99–162. Interscience, New York.

—— and PERLMANN, G. (1938). Hydrogenation of vitamin B_1 and other quaternary thiazoles. *J. Am. Chem. Soc.* **60**:2574–2578.

LIPSCHITZ, M. A., POTTER, V. R., and ELVEHJEM, C. A. (1938). The mechanism of the enzymatic synthesis of cocarboxylase. *J. Biol. Chem.* **124**:147–150.

LOHMANN, K., and SCHUSTER, P. (1937). Untersuchungen über die Cocarboxylase. *Biochem. Z.* **294**:188–214.

LUTWAK-MANN, C. (1938). Alcohol dehydrogenase of animal tissues. *Biochem. J.* **32**:1364–1374.

LYMAN, C. M., and BARRON, E. S. G. (1937). Studies on biological oxidations. VIII. The oxidation of glutathione with copper and hemochromogens as catalysts. *J. Biol. Chem.* **121**:275–284.

MACMUNN, C. A. (1887). Further observations on myohaematin and the histohaematins. *J. Physiol.* **8**:51–65.

MICHAELIS, L. (1937). Review of the semiquinone problem. *Trans. Electrochem. Soc.* **71**:107–125.

—— (1940). Occurrence and significance of semiquinone radicals. *Ann. N. Y. Acad. Sci.* **40**:39–76.

—— and FRIEDHEIM, E. A. H. (1931). Potentiometric studies on complex iron systems. *J. Biol. Chem.* **91**:343–353.

—— and MENTEN, M. L. (1913). Die Kinetik der Invertinwirkung. *Biochem. Z.* **49**:333–369.

——, SCHUBERT, M. P., and SMYTHE, C. V. (1936). Potentiometric study of the flavins. *J. Biol. Chem.* **116**:587–607.

NEGELEIN, E., and GERISCHER, W. (1936). Verbesserte Methode zur Gewinnung des Zwischenferments aus Hefe. *Biochem. Z.* **284**:289–296.

—— and HAAS, E. (1935). Über die Wirkungsweise des Zwischenferments. *Biochem. Z.* **282**:206–220.

—— and WULFF, H. J. (1937). Diphosphopyridineproteid, Alkohol, Acetaldehyd. *Biochem. Z.* **293**:351–389.

OCHOA, S. (1941). "Coupling" of phosphorylation with oxidation of pyruvic acid in brain. *J. Biol. Chem.* **138**:751–773.

—— (1944). α-Ketoglutaric dehydrogenase of animal tissues. *J. Biol. Chem.* **155**: 87–100.

PETERS, R. (1898). Über Oxydations- und Reduktionsketten und den Einfluss komplexer Ionen auf ihre elektromotorische Kraft. *Z. physik. Chem.* **26**: 193–236.

POTTER, V. R. (1941). Studies on the mechanism of hydrogen transport in animal tissues. III. Cyanide inhibition of cytochrome *c* reduction. *J. Biol. Chem.* **137**:13–20.

PREISLER, P. W. (1930). Oxidation-reduction potentials and the possible respiratory significance of the pigment of nudibranch *Chromodoris zebra*. *J. Gen. Physiol.* **13**:349–359.

PULVER, R., and VERZÁR, F. (1939). Die Phosphorylierung von Riboflavin durch Darmschleimhaut. *Enzymologia* **6**:333–336.

QUASTEL, J. H., and WHETHAM, M. D. (1924). The equilibria existing between succinic, fumaric, and malic acids in the presence of resting bacteria. *Biochem. J.* **18**:519–534.

—— and WOOLDRIDGE, W. R. (1928). Some properties of the dehydrogenating enzymes of bacteria. *Biochem. J.* **22**:689–702.

RICE, O. K. (1936). On the zero-point energy of an activated complex and the reaction $2\ NO + O_2 = 2\ NO_2$. *J. Chem. Phys.* **4**:53–59.

RIEGEL, B., SMITH, P. G., and SCHWEITZER, C. E. (1940). The oxidation-reduction potential of vitamin K_1. *J. Am. Chem. Soc.* **62**:992.

SCHLENK, F. (1942). Identification of the carbohydrate group in the nicotinamide nucleotides. *J. Biol. Chem.* **146**:619–625.

SILVERMAN, M., and WERKMAN, C. H. (1939). Bacterial synthesis of cocarboxylase. *Enzymologia* **5**:385–387.

—— and —— (1941). The formation of acetylmethylcarbinol from pyruvic acid by a bacterial enzyme preparation. *J. Biol. Chem.* **138**:35–48.

STERN, K. G. (1935). Oxidation-reduction potentials of toxoflavin. *Biochem. J.* **29**: 500–508.

STOTZ, E., SIDWELL, A. E., JR., and HOGNESS, T. R. (1938). The spectrophotometric determination of the equilibrium in oxidation-reduction systems; the potential of cytochrome *c*. *J. Biol. Chem.* **124**:11–23.

STRAUB, F. B. (1939). Isolation and properties of a flavoprotein from heart muscle tissue. *Biochem. J.* **33**:787–792.

SUMNER, J. B., and DOUNCE, A. L. (1937). Crystalline catalase. *J. Biol. Chem.* **121**:417–424.

—— and —— (1939). Catalase. II. *J. Biol. Chem.* **127**:439–447.

——, ——, and FRAMPTON, V. L. (1941). Catalase. III. *J. Biol. Chem.* **136**: 343–356.

SZENT-GYÖRGYI, A. (1928). Observations on the function of peroxidase systems and the chemistry of the adrenal cortex. *Biochem. J.* **22**:1387–1409.

—— (1931). On the function of hexuronic acid in the respiration of the cabbage leaf. *J. Biol. Chem.* **90**:385–393.

—— (1937). *Studies on Biological Oxidation and Some of Its Catalysts.* Renyi, Budapest, p. 74.

—— (1937a). Oxidation and fermentation. In *Perspectives in Biochemistry*, pp. 165–174. Cambridge Univ. Press, London.

TADOKORO, T., and TAKASUGI, N. (1939). The chemical properties of ascorbic acid oxidase. III. *J. Chem. Soc. Japan* **60**:188–190.

THEORELL, H. (1936). Reines Cytochrom *c*. II. Mitteilung: Darstellung, Eigenschaften, Ionenbeweglichkeit, Diffusion und Absorptionsspektrum des Cytochroms *c*. *Biochem. Z.* **285**:207–218.

—— (1937). Die freie Eiweisskomponente des gelben Ferments und ihre Kupplung mit Lactoflavinphosphosäure. *Biochem. Z.* **290**:293–303.

—— (1940). Reversible Spaltung einer Peroxydase. *Arkiv Kemi, Mineral. Geol.* **B14**, No. 20:1–3.

—— (1942). Crystalline peroxidase. *Arkiv Kemi, Mineral. Geol.* **A16**, No. 2:1–11.

—— (1942a). The magnetic properties of crystalline horse-radish peroxidase and some of its derivatives. *Arkiv Kemi, Mineral. Geol.* **A16**, No. 3:1–11.

—— and ÅKESSON, Å. (1941). Studies on cytochrome *c*. I. Electrophoretic purification of cytochrome *c* and its amino acid composition. *J. Am. Chem. Soc.* **63**:1804–1811.

—— and SWEDIN, B. (1939). Mangan als Aktivator der Dioxymaleinsäureoxydase. *Naturwissenschaften* **27**:95–96.

WARBURG, O. (1926). Über die Wirkung des Kohlenoxyds auf den Stoffwechsel der Hefe. *Biochem. Z.* **177**:471–486.

—— (1932). Das säuerstoffübertragende Ferment der Atmung. *Z. angew. Chem.* **45**:1–6.

—— and CHRISTIAN, W. (1932). Über ein neues Oxydationsferment und sein Absorptionsspektrum. *Biochem. Z.* **254**:438–458.

—— and —— (1933). Über das gelbe Oxydationsferment. *Biochem. Z.* **257**:492.

—— and —— (1933a). Über das gelbe Ferment und seine Wirkungen. *Biochem. Z.* **266**:377–411.

—— and —— (1938). Co-Ferment der d-Alanin-Oxydase. *Biochem. Z.* **296**:294.

—— and —— (1938a). Isolierung der prosthetischen Gruppe der d-Aminosäureoxydase. *Biochem. Z.* **298**:150–168.

—— and —— (1939). Proteinteil des kohlenhydrat-oxydierenden Ferments der Gärung. *Biochem. Z.* **301**:221–222.

—— and —— (1939a). Isolierung und Kristallisation des Proteins des oxydierenden Gärungsferments. *Biochem. Z.* **303**:40–68.

WEIJLARD, J., and TAUBER, H. (1938). Synthesis, isolation and identification of cocarboxylase. *J. Am. Chem. Soc.* **60**:2263–2264.

WEISS, J., and WEIL-MALHERBE, H. (1939). Decomposition of hydrogen peroxide by catalase. *Nature* **144**:866.

WIELAND, H. (1932). *On the Mechanism of Oxidation.* Yale Univ. Press, New Haven.

WURMSER, R., and FILITTI-WURMSER, S. (1938). Le potentiel d'oxydoréduction du cytochrome. *Compt. rend. soc. biol.* **127**:471–474.

YAKUSHIJI, E., and MORI, T. (1937). Untersuchungen über das Cytochrom b. Isolierung, Eigenschaften und seine Rolle im Reaktionsmechanismus der Zellatmung. *Acta Phytochim. Japan* **10**:113–123.

—— and OKUNUKI, K. (1941). Isolierung der a-komponente des Cytochroms und ihre Eigenschaften. *Proc. Imp. Acad. Tokyo* **17**:38–40.

CHAPTER VII

OXIDIZING ENZYME SYSTEMS OF WHEAT AND FLOUR

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INTRODUCTION

The oxidizing enzymes play an essential part in the respiration and germination of the cereal grains and in the processes of alcoholic fermentation. Pasteur, in one of his brilliantly simple experiments, showed that fermentation is really anaerobic respiration, and Meyerhof's experiments proved that the rate at which sugar is decomposed by yeast is less in the presence than in the absence of air or oxygen. This so-called "Pasteur effect," and the respiration cycle, have been the subject of numerous researches. It is not difficult to ascertain single facts bearing on the respiration or fermentation mechanisms, but if such facts are not considered in relation to the whole subject they are likely to obscure rather than to clarify our concepts.

Since foods are utilized by means of reactions of the oxidizing enzyme systems, the knowledge of their mechanism has an important bearing on nutrition. Because of the widespread occurrence of oxidizing enzymes and their important functions, the interrelation of oxidation-reduction reactions and their application to the problems of the cereal chemist, from the growing of grains to their ultimate use as food, constitute a fundamental and fruitful field of research.

The general nomenclature of the oxidizing enzyme systems employed by Barron will be used in this chapter. Oxidation is defined, according to W. M. Clark, as the withdrawal of electrons with or without the loss of hydrogen, and reduction as the addition of electrons with or without the addition of oxygen. The confusion in the literature caused by arbitrary and rigid distinctions between oxidases and dehydrogenases can thus be

avoided. Nevertheless, in writing a review there is a real difficulty because, in the current as well as in the past literature, certain oxidizing enzymes are usually called dehydrogenases. As Barron has indicated in Chapter VI, this term is unfortunate since the enzyme itself does not remove hydrogen from the substrate. It has been thought best, however, to retain the term "dehydrogenase" where the authors themselves have used it.

A discussion of oxidizing enzymes necessitates a consideration of systems rather than individual enzymes because the transfer of electrons from the substrate to oxygen takes place through the medium of various coenzymes and reversible systems. In this chapter the systems are classified as: iron–porphyrin, copper–protein, phosphopyridine nucleotide, flavin systems, and other "dehydrogenases." Oxalase and lipoxidase are treated separately. And the final section deals more specifically with the oxidation-reduction systems in flour and dough.

IRON–PORPHYRIN ENZYME SYSTEMS

Catalase. Catalase is an iron–porphyrin compound and is of value in regulating oxidations. The sole reaction effected by catalase is the decomposition of hydrogen peroxide to water and oxygen. Catalase may be estimated by measuring the oxygen liberated, or the amount of hydrogen peroxide not decomposed within a prescribed time interval. Catalase is inactivated by hydrocyanic acid and hydrogen sulfide. No work has been done on the constitution of plant catalases, but Sumner and Dounce (1937, 1937a, 1939) have crystallized catalase from beef liver. It has been shown by calculation that the crystalline catalases contain four hematin groups.

The catalase activity of wheat products has been studied by many early investigators, such as Wender and Lewin (1904), Wender (1905), Liechti (1909), Miller (1909), Bailey (1917), Marion (1920), Lindet (1920), Merl and Daimer (1921), Fernández and Pizarroso (1921), Bornand (1921), and Marotta and Kaminka (1922). These investigators have shown that catalase activity is lowest in the endosperm and highest in the bran and peripheral layers of the wheat kernel, and that it increases progressively as the grade of flour decreases. Moreover, since catalase activity parallels the ash content of milling separations but increases at a much more rapid rate, it has been suggested that catalase measurements might serve as a useful index of the grade of flours. But Marotta and Kaminka (1922) and Blish and Bode (1935) have shown that, among flours of a comparable grade, the catalase activity varies widely in accordance with the origin of the wheat. Canadian flours examined by Blish and Bode had nearly four times as much catalase as flours of a similar grade from Kansas and Texas, and spring wheat varieties gave higher values than winter wheats grown under

the same climatic conditions. Prokopenko (1927) also found higher catalase activity in spring than in winter wheat, and Ivanoff (1932) reported relatively higher catalase activity in wheats grown in northern or mountainous regions of Russia. Blish and Bode (1935) stated that, if catalase activity were used as a criterion of grade, a definite knowledge of the locality from which the wheat originated would be essential.

The work of Blish and Bode also showed that the catalase activity of flour was lowered by grinding the flour in a ball mill, and that the activity of this enzyme was also reduced significantly by commercial bleaching agents, such as nitrogen trichloride and benzoyl peroxide, and by natural aging at room temperature. It had previously been indicated by Gelissen (1924) that chlorine decreased the action of catalase on hydrogen peroxide. Van der Lee (1932) found that chlorates, perchlorates, bromates, iodates, periodates, and persulfates inhibited the action of the catalase of flour. It was postulated by Elion (1942) that the bleaching of flour by hydrogen peroxide might be prevented by the catalase of the flour. He found that small amounts of several reducing agents (0.2 per cent based on the flour weight) greatly retarded the decomposition of hydrogen peroxide by the catalase of flour. Among these reducing agents were *l*-ascorbic acid, maleic acid, stannous chloride, pyrocatechin, carminic acid, and formaldehyde. The inhibitory action of *l*-ascorbic acid on the catalase of flour decreased progressively as smaller amounts of this reducing agent were employed, and a method was thus provided for adjusting the catalase activity of various flours to the same level. Prokoshev, Margolina, and Babichev (1936) found that treatment with hydrogen sulfide caused a slight decrease of the catalase activity of dormant wheat and a considerable decrease in that of germinating wheat.

Observations by Bach and Oparin (1923) showed that the catalase activity of wheat increased during germination and then decreased; maximum activity was reached in three to four days with the experimental conditions employed. Knyaginichev and Palilova (1940) found that each stage of development in the growing wheat plant is characterized by changes in the activity, temperature coefficient, and energy of activation of catalase. These changes are probably due to the presence of substances which modify the activity of catalase.

Bunzell and co-workers (1930, 1932) have used a determination of the catalase activity of the rope organism (*Bacillus mesentericus*) as a measure of the degree of rope infection of bread. These investigators designed an apparatus suitable for testing bread crumb wherein the amount of oxygen, liberated in the presence of hydrogen peroxide and the bread crumb, indicated the amount of rope infection.

Catalase probably guards against harmful accumulation of hydrogen

peroxide during the respiratory processes of grains and in other processes in which an excess of hydrogen peroxide might inhibit the normal oxidation-reduction reactions.

Peroxidase. Peroxidase is another iron–porphyrin compound which, in the presence of hydrogen peroxide, can oxidize a number of aromatic amines and phenols, such as *o*-phenylenediamine, benzidene, hydroquinone, pyrogallol, and guaiacol. Most plants contain peroxidase. The method most generally employed for measuring its activity is that of Willstätter, which consists in the oxidation of pyrogallol and the determination of the resulting purpurogallin by titration or by comparison with color standards.

The presence of peroxidase in wheat was first demonstrated by Bertrand and Muttermilch (1907), who showed that hydroquinone could be oxidized by an extract of wheat bran. According to Prokopenko (1928), spring wheats possess more peroxidase activity than winter wheats. Bach and Oparin (1923) germinated wheat from the 1919 and 1921 crops and measured the peroxidase activity as the germination proceeded. They employed the method of Willstätter, and referred their results to 100 milligrams of total nitrogen of the kernel. Peroxidase activity was found to increase during germination, reaching its maximum in eight days. Bach and Oparin state that peroxidase and other oxidizing enzymes are present in both resting and germinating wheat in much higher amounts than the hydrolytic enzymes (amylases and proteases), but this conclusion might prove erroneous if the more accurate methods recently developed were used to determine the amylases and proteolytic enzymes. The peroxidase, catalase, amylase, and proteolytic activities of wheat samples taken at intervals during the growing period were studied by Bach, Oparin, and Vener (1926). The curves for enzyme activity as a function of time increased irregularly, reached a maximum, and then decreased. During the course of ripening, two opposite changes were alleged to take place simultaneously: the formation of active enzymes, and their transition into inactive zymogens—the latter can change into enzymes when the seed germinates.

The distribution of peroxidase in wheat and other cereal grains has not been studied. The few color tests reported in the literature indicate that bran contains a greater quantity than other fractions of the kernel.

Peroxidase occurs in all grains, and it probably functions in oxidizing certain phenols and amines, which may be harmful to the plant under certain conditions. There is little direct evidence on this point.

Only one reference to the effect of peroxidase on the baking quality of flour has been found. Blagoveshchenskiĭ (1939–40) reported that satisfactory bread was made from badly spoiled flour by adding the following mixture: 0.2 per cent lactic acid, 0.1 per cent hydrogen peroxide, and per-

oxidase. The same effect might have been achieved by Agene or bromate since the beneficial effect was apparently due to oxidation.

Cytochrome Oxidase. A number of investigators have theorized on the role of the oxidizing enzymes in plant respiration. The basis of many conflicting conjectures was the data obtained by the use of certain color tests, but such tests can be misleading and need to be interpreted with caution. It has been only comparatively recently that our knowledge of the constitution of some of the oxidizing enzymes and the probable mechanism of the oxidation-reduction systems in plant and animal cells has been clarified. The researches of Warburg and Keilin and Hartree have been outstanding in elucidating the enzyme mechanism of the respiration cycle. The cytochromes, called *a*, *b*, and *c*, are iron–porphyrin proteins and can be differentiated by their characteristic absorption bands. Warburg's oxygen-transferring enzyme, previously known as indophenol oxidase, is now considered to be cytochrome *c* oxidase. This enzyme was formerly measured by the "nadi test," the formation of indophenol blue from α-naphthol and *p*-phenylenediamine. More recent methods are based on the photosensitive inhibition of respiration by carbon monoxide, which is catalyzed by the enzyme. The cytochrome system is made up of the cytochromes and cytochrome *c* oxidase. Cytochrome oxidase catalyzes the oxidation of nonautoxidizable cytochromes and is, in turn, oxidized by molecular oxygen. The cytochrome system, although still only partially understood, is of great importance in oxidizing many substrates in both plant and animal cells.

Interesting experimental data on the cytochrome oxidase of wheat germ have been recently presented by Brown and Goddard (1941). Extracts were prepared from wheat germ and the presence of cytochrome oxidase was demonstrated by the cytochrome *c* stimulation of the oxidation of hydroquinone, *p*-phenylenediamine, and dihydroxyphenylalanine. The oxidase was shown to be heat labile; the activity of the preparation from wheat germ was half destroyed at 55.5°C and entirely eliminated after heating 15 minutes at that temperature. The cytochrome oxidase preparation was found to be inhibited by hydrocyanic acid, sodium azide, and carbon monoxide, with reversal of the carbon monoxide inhibition by visible light. A major fraction of the respiration of intact wheat embryos (dissected from the same wheat) was also shown to be inhibited by hydrocyanic acid and sodium azide, and by carbon monoxide with reversal by light of the carbon monoxide inhibition.

The first attempts to extract cytochrome *c* from wheat germ gave questionable results. Recently, however, cytochrome *c* has been isolated from wheat embryos in the quantity of about 4.6 milligrams per kilo of dry weight (Goddard, 1944). The cytochrome *c* has the same absorption spec-

trum as heart cytochrome *c* and similar catalytic properties. The method of extraction and preparation of cytochrome oxidase from wheat germ has been much improved and simplified since the original description given in the paper by Brown and Goddard (1941). The preparation is reproducible and has a Q_{O_2} of approximately twenty. This value is low compared with that of preparations from animal sources but high in comparison with that of extracts from other plant materials; it apparently indicates nearly complete extraction of the enzyme.

COPPER–PROTEIN ENZYME SYSTEMS

Tyrosinase. Tyrosinase is a copper–protein compound and, like other metalloprotein enzymes, depends on the metal ion for transfer of electrons to molecular oxygen. Tyrosinase catalyzes the oxidation of several phenolic substances such as phenol, *m*- and *p*-cresol, catechol, and tyrosine. Earlier measurements of its activity were based on the color formed with phenol or tyrosine as substrates, but now the enzyme activity is usually determined by measuring the oxygen uptake in a Warburg apparatus. The optimum hydrogen-ion concentration varies between *p*H 5.5 and 8.0 depending on the buffer and the substrate.

Bertrand and Muttermilch (1907) were the first to demonstrate the presence of tyrosinase in wheat. The enzyme was prepared by alcoholic precipitation of a centrifuged water extract of bran, and its activity was found to be destroyed by heating its solution for 5 minutes at 100°C. Little is known about the quantitative distribution of tyrosinase in the wheat kernel or in other grains, but it seems safe to assume that, as with so many other oxidases, the largest amounts occur in the outer layers of the grain.

Experiments by Brown and Goddard (1941) indicated that no thermolabile polyphenol oxidase was present in extracts of wheat germ.

The color produced by the action of certain oxidizing enzymes on various dyes has been suggested as a means of differentiating wheat varieties. For this purpose Voss (1938) worked out a system based on color tests for the tyrosinase, peroxidase, and dehydrogenase activities of wheats. According to Voss, the tyrosinase activity of the grain and bran of individual varieties shows only a slight range with the origin and the growing conditions. With phenol as a substrate, there is a marked variation in the color produced when varieties of wheat are compared. The rate of melanin (black pigment) formation was found to increase with temperature. Apparently the tyrosinase content of wheats is an inherited characteristic of the variety. Phenol color reactions were also employed by Fraser and Gfeller (1936) as a means of studying inheritance in varieties of hard spring

wheat. The kernels were soaked in water for 16 hours, drained, allowed to dry for 1 hour, immersed in 1 per cent phenol solution for 4 hours, and then dried on blotting paper. Varieties of Canus, Garnet, Marquis, Red Fife, Reward, and Ruby, harvested at two-day intervals from the soft dough stage to maturity, gave intensified color reactions with phenol as they approached maturity. These authors believe that the presence of two genetically linked factors may afford a possible explanation of the inheritance of phenol color reactions of spikes and kernels in crosses of Garnet and Red Fife. Miczyński (1938) also studied the genetics of the phenol color reaction in wheat.

Ascorbic Acid Oxidase. Ascorbic acid oxidase is considered to be a copper–protein which acts directly on *l*-ascorbic acid to form dehydroascorbic acid. The activity of this enzyme may be measured by oxygen uptake in a Warburg apparatus, or by titration of the remaining ascorbic acid with 2,6-dichlorophenolindophenol.

Jørgensen (1935) reported that *l*-ascorbic acid could act as a flour improver. Because ascorbic acid is a reducing agent, whereas most flour improvers are oxidizing agents, the subject elicited much interest among cereal chemists. Melville and Shattock (1938) showed that dehydroascorbic acid is a more effective improver than ascorbic acid and that the oxidized form of *l*-ascorbic acid is actually responsible for its beneficial effect on flour. They demonstrated that flour contains an enzyme (presumably ascorbic acid oxidase) capable of catalyzing the oxidation of ascorbic acid to dehydroascorbic acid. Using a centrifuged flour extract and ascorbic acid, they found that the added ascorbic acid was oxidized. Proskuryakov and Pavlinova (1940) also found that ascorbic acid and, better still, dehydroascorbic acid, when added to certain flours, effectively improved the quality of the bread. According to these investigators, the added ascorbic acid is oxidized by an oxidase present in flour, and glutathione exerts a protective action in this oxidation. Investigations by Feaster and Cathcart (1941) show that *d*-isoascorbic acid is ineffective as a dough improver.

PHOSPHOPYRIDINE NUCLEOTIDE AND FLAVIN SYSTEMS

All enzymatic oxidation-reduction reactions consist in electron transfers, and in many cases the enzymes and coenzymes involved are hydrogen transfer systems. The protein components of such systems are often referred to as dehydrogenases. Their activity can be measured by the Thunberg technique using some reversible dye such as methylene blue or thionine, or by the rate of oxygen uptake in the presence of the coenzyme in a Warburg or Barcroft apparatus (Dixon, 1943).

Hexosediphosphate Dehydrogenase. Using the time of color removal of methylene blue as an index, Neuenschwander (1928) reported that flour alone has only a very slight effect on the decoloration of methylene blue. With the addition of hexose phosphate (a hydrogen donator), there is even a slight retardation; and with diphosphopyridine nucleotide (DPN) alone, the action is not changed significantly. But if both the coenzyme and hexose phosphate are added, the activity of the enzyme is increased enormously. For example: where flour, buffer, and methylene blue were used, the time required to decolorize the dye was 21 to 23 hours; with coenzyme added, the time was 18 hours; with hexose phosphate alone, it was 24 hours; but with both coenzyme and hexose phosphate, the time required for decolorization was shortened to 101 minutes. The poorer the grade of the flour, the greater is the speed of color removal. Various wheats showed differences in their dehydrogenase content as determined by the speed of decolorization; Manitoba (Western Canadian hard red spring wheat) showed the highest amount.

Variety	Time of decolorization, hr
Manitoba	$2^3/_4$
Rose Fe	$3^3/_4$
Northern Spring	$4^1/_2$
Australian	$5^1/_2$
Swedish	$7^1/_2$ to 8

Andersson (1932) also demonstrated the presence of a dehydrogenase, which decolorized methylene blue in the presence of hexose phosphate, in a number of seeds for which Thunberg (1931) had reported negative results. The inactivity of such seeds was not due to the absence of the enzyme, but rather to an inadequate supply of coenzyme. During the first few days of germination of wheat, rye, and oats, the activity of the dehydrogenase increases considerably. The increase is not due, however, to a greater enzyme content, but to the formation of increased amounts of DPN. Andersson measured the amount of DPN necessary to obtain the maximum action of the hexosediphosphate dehydrogenase. It should be noted, however, that recent research seems to indicate that there is no true hexosediphosphate dehydrogenase, but that the enzyme is really 1,3-diphosphoglyceraldehyde dehydrogenase. It is believed that the hexose diphosphate employed by the earlier investigators was converted into the phosphoglyceraldehyde before undergoing oxidation.

Malate and Glutamic Acid Dehydrogenases. Andersson (1933, 1934), again measuring the fading of methylene blue in the presence of coenzyme, found malate and glutamic acid dehydrogenases in wheat. Other oxidases are probably present also.

Fumaric, Succinic, and Formic Acid Dehydrogenases. The occurrence in wheat germ of fumaric, succinic, and formic acid dehydrogenases was reported by von Euler and Malmberg (1936). A system consisting of specified amounts of methylene blue, dialyzed extract of wheat germ, phosphate buffer (*p*H 7.6), flavin enzyme, DPN, and fumarate, was decolorized in 25 minutes at 30°C; without DPN and flavin, decolorization required 165 minutes. The oxidation-reduction process is catalyzed in phosphoric acid but does not take place when Ringer's solution is employed in making the extract. Succinic dehydrogenase has also been found by Goddard (1944) to occur in wheat germ in low but measurable amounts. The enzyme is very labile and loses 50 per cent of its activity in 24 hours.

OXALASE

Zalesskiĭ and Kukharkova (1928) found that the velocity of aerobic and anaerobic oxidation of oxalic acid by wheat (as measured by the carbon dioxide produced) is unaffected by the addition of methylene blue but is inhibited by quinone and hydroquinone. The inhibiting effect of the quinones was thought to be due to their greater affinity for oxygen; this diminishes the supply available for the oxidation of oxalic acid. The authors conclude that oxalase is a dehydrogenase that can use only molecular oxygen as a hydrogen acceptor. This conclusion seems doubtful since no oxidases have been found where molecular oxygen is the only oxidizing agent.

OTHER DEHYDROGENASES

According to Kretovich and Sokolova (1942), the damage to grain stored under unfavorable conditions or over long periods of time is due in part to the activity of the oxidation-reduction enzymes of the embryo, especially the dehydrogenases. These investigators found that the dehydrogenases of the wheat embryo are only slightly active in acid medium and are completely inactive at *p*H 4.5 to 5.0. The optimum activity was found to be at *p*H 7.2 to 7.5 when McIlvain's buffers were employed and at *p*H 7.3 to 9.2 when Sørensen's phosphate buffers were used. The optimum temperature was observed at 50°C. As might be expected, the enzyme activity was considerably increased in the presence of glutamic acid and hexose phosphate.

The activity of the oxidizing enzymes in wheat and its milled products has been demonstrated mainly by qualitative color tests. Measurement of oxygen uptake should provide more accurate quantitative data. Positive statements concerning the role of these catalysts in respiration and germi-

nation and their function in alcoholic fermentation must await further evidence. Recent work by Berger and Avery (1943) on the coleoptile of oats proves that auxin, the plant growth hormone, greatly stimulates the action of alcohol dehydrogenase. This work suggests the possibility that alcohol dehydrogenase is an important factor in the control of plant growth.

LIPOXIDASE

Practically all the work on lipoxidase, which oxidizes fats and carotenoid pigments, has been done with soybean meal. But its presence has been demonstrated in wheat germ, though only to the extent of 810 units per gram of dry weight as compared with 35,000 units for soybean meal (Sumner, 1943). The enzyme is important because it accelerates the development of rancidity in fat, and because it causes losses of carotene, vitamin A, and ascorbic acid. It is also worth noting that soybean meal has been employed by Haas and Bohn (1934) for bleaching the xanthophyll and carotenoid pigments of flour doughs.

Methods for the determination of lipoxidase have been based both on peroxide formation from fat and on the oxidation of carotene. In dilute aqueous suspensions, the fat peroxides oxidize ferrous iron to ferric iron, and the latter can be determined colorimetrically as the thiocyanate (Sumner, 1943). A direct colorimetric method has been used by Balls, Axelrod, and Kies (1943) to follow the rate of oxidation of carotene, or of leuco dyes such as leuco-*o*-chlorophenolindophenol. Certain leuco dyes have also been used by Van Fleet (1942, 1942a, 1943) to demonstrate *in vivo* the location and function of the unsaturated fat oxidase in specific tissues of a number of crop plants.

The history of our knowledge of lipoxidase is comparatively brief. In 1931, Hauge and Aitkenhead reported that they had found a substance in alfalfa that could destroy carotene. At about the same time, André and Hou (1932) demonstrated that an enzyme present in soybeans could oxidize the fat. Experiments by Sumner and Sumner (1940) and Tauber (1940) proved that the so-called "carotene oxidase" found in soybeans could oxidize carotene only in the presence of unsaturated fats. Sumner (1942a) showed that carotene is not oxidized, either directly or indirectly, by the peroxides formed from the unsaturated fat, but that the oxidation of carotene demands that the peroxidation of the unsaturated fat be actually in progress. Strain (1941) and Sumner (1942) believe that carotene is oxidized by an intermediate or nascent product resulting from the oxidation of the unsaturated fat linkages. Working with soybean lipoxidase, Balls, Axelrod, and Kies (1943) demonstrated that only linoleic, linolenic, and arachidonic acids were oxidized, and that oleic acid was not attacked. These

investigators also found that purothionin, isolated from wheat flour (Balls, Hale, and Harris, 1942) inhibits the oxidation. When more fat was used as substrate, more purothionin was required for inhibition. Purothionin seems to act by inhibiting the initial fat oxidation.

As the bleaching of flour on natural aging and rancidity development in flour and feeds is doubtless due, at least in part, to the action of lipoxidase, further investigations of the activity and distribution of this enzyme in the wheat kernel deserve attention.

OXIDIZING ENZYME SYSTEMS OF YEAST

Certain of the oxidizing enzymes of yeast influence the course of fermentation and are also affected by small quantities of oxidizing and reducing substances. Yeast fermentation is reviewed in Chapter XI and will not be discussed in any detail here. When glucose is fermented by yeast, a phosphorylation takes place, and one molecule of phosphoric acid is usually esterified for each molecule of carbon dioxide formed. During the process of alcoholic fermentation, many coupled oxidation-reduction reactions occur and some oxidizing enzyme systems are involved. Yeast, as well as many plants, contains carboxylase (a diphosphothiamine protein) that decarboxylates α-keto acids to aldehydes and carbon dioxide. Pyruvate mutase is another diphosphothiamine protein that functions in fermentation, forming lactic acid and acetic acid from pyruvic acid. Bakers' yeast contains an alcohol dehydrogenase that acts on ethyl alcohol to form acetaldehyde; activity is enhanced by sulfhydryl groups and DPN functions in this system. Yeast also contains lactic acid dehydrogenase that can convert α-hydroxy acids to α-keto acids. Glutamic acid dehydrogenase is present in yeast and exerts its activity by converting glutamic acid to α-ketoglutaric acid; triphosphopyridine nucleotide (TPN) is required for this enzyme. So far as the writer is aware, there is no information on the function of glutamic acid dehydrogenase in alcoholic fermentation.

Wheat flours (especially patent grades) are low in DPN, which must be present in order that certain of the oxidizing systems may function. Accordingly, it is possible that the dehydrogenases of flour do not exert their maximum efficiency unless additional DPN is provided—for instance, by the yeast added in making up a dough.

OXIDATION-REDUCTION SYSTEMS IN FLOUR AND DOUGH

It is known that all wheat products have an oxidation-reduction potential which may be characterized as reducing. Flour shows a potential

less than that of bran and much less than that of germ. Potel and Chaminade (1935) found a rather narrow range of E_h, $+0.08$ to $+0.12$ volt, at pH 6.2 for the potential of flours, and a value of -0.169 volt at pH 6.2 for an extract of wheat germ. Yeast suspensions have an oxidation-reduction potential in the range of that of germ extracts; in other words, they are rather strongly reducing. Sullivan, Howe, Schmalz, and Astleford (1940), using potentiometric titrations, found that low-grade flour possessed more reducing material than clear flour and that clear contained more than patent flour. It was likewise observed by Freilich (1941) that lower-grade flours contained more reducing matter than the more highly purified grades, and that Northwest flours showed more reducing material than Texas flours. Experiments by Shen and Geddes (1942) demonstrated that the reducing matter of a nonfermenting dough (octyl alcohol treated) increased to a greater extent with time than did that of a fermenting dough. Previous work by Sullivan, Howe, and Schmalz (1936), and Sullivan and Howe (1937) proved that glutathione (an activator of many enzymes) was largely responsible for the strong reducing activity of germ and for the harmful effect of germ in baking. Laitinen and Sullivan (1941), using the dropping mercury electrode, found that potassium chloride extracts of germ and bran gave pronounced anodic waves (indicating an electrooxidizable substance) occurring at the same potential as glutathione. The relative heights of the waves indicated a much higher concentration of the material in germ than in bran. No reducing substance could be demonstrated in extracts prepared by either a potassium chloride or acetate buffer (pH 4.7) extraction of flour. Sullivan *et al.* (1940) state that there is a reducing medium in dough of such strength that it is able to reduce sulfur to hydrogen sulfide.

For years it has been known that many flours do not exhibit their optimum baking properties until they have been aged or treated with certain oxidizing agents. There is a certain optimum oxidation-reduction potential at which every flour will give its best results in breadmaking; this may vary with the gluten quality. It is generally accepted that flours of longer extraction need more oxidation than short patents.

Chemists have sought for some time an explanation of the mechanism of the action of certain oxidizing agents on flour. While some investigators have felt that such compounds act by inhibiting the proteolytic activity of flour, others have maintained with equal force that the action has little to do with proteolytic activity. The literature on this subject has been thoroughly reviewed by Shen and Geddes (1942), and it appears that neither side has proved its case (see also Chapter IX). Natural aging, or improvers such as nitrogen trichloride, bromate, iodate, or dehydroascorbic acid, apparently function by oxidizing a reducing group of a protein or a peptide of lower molecular weight, and thus cause desirable changes in the

colloidal properties of the gluten. Reducing substances, such as hydrocyanic acid, cysteine, and glutathione, harm flour by shortening the mixing time and producing soft, sticky doughs with poor carbon dioxide retention. Hence, there must be one or more oxidation-reduction systems present. The sulfhydryl group is doubtless involved, but whether it is a constituent part of a definite enzyme, either proteolytic or oxidizing, or whether some sulfur-containing compound is adsorbed on the gluten or exists as a constituent part of it, remains to be determined. Stern (1944) assumes the aging and maturing of dough is connected with the oxidation of the glutathione of germ particles contained in the flour and that the oxidation of sulfhydryl groups in stored flour and fermenting dough may be attributed to dehydrogenases. Since enzymes themselves are proteins and since it is difficult to destroy the enzymes in flour without modifying the gluten in some degree, it is hard to prove conclusively whether the observed oxidation-reduction reactions are due to inhibition or activation of an enzyme system, or to a direct action on some linkage of a substance which is not an enzyme. The mechanism of the action of improvers still remains to be discovered. Oxidizing enzymes containing the sulfhydryl groups, or activated by it, may be involved in the natural and artificial aging of flour, and future work on the oxidizing enzyme systems might shed some light on this intriguing and complex problem.

BIBLIOGRAPHY

ANDERSSON, B. (1932). Über Hexosediphosphatdehydrogenase und Carboxylase in Pflanzensamen. *Z. physiol. Chem.* **210**:15–29.

—— (1933). Über Co-Zymaseactivierung einiger Dehydrogenasen. *Z. physiol. Chem.* **217**:186–190.

—— (1934). Die Co-Zymase als Co-Enzym bie enzymatischen Dehydrierungen. *Z. physiol. Chem.* **225**:57–68.

ANDRÉ, E., and HOU, K. W. (1932). Sur la présence d'une oxydase des lipides ou lipoxidase dans la graine de soja, *Glycine soja*. *Compt. rend.* **194**:645–647.

BACH, A., and OPARIN, O. (1923). Über die Fermentbildung in keimenden Pflanzensamen. *Biochem. Z.* **134**:183–189.

——, ——, and VENER, R. A. (1926). Quantitative variations of enzymes in grains of wheat in the course of ripening, resting and germinating. *Trans. Karpov. Chem. Inst.* **5**:62–70. (*Chem. Abstracts* **22**:2186.)

BAILEY, C. H. (1917). The catalase activity of American wheat flours. *J. Biol. Chem.* **32**:539–545.

BALLS, A. K., AXELROD, B., and KIES, M. W. (1943). Soybean lipoxidase. *J. Biol. Chem.* **149**:491–504.

——, HALE, W. S., and HARRIS, T. H. (1942). A crystalline protein obtained from a lipoprotein of wheat flour. *Cereal Chem.* **19**:279–288.

——, ——, and —— (1942a). Further observations on a crystalline wheat protein. *Cereal Chem.* **19**:840–844.

BERGER, J., and AVERY, G. S., JR. (1943). The mechanism of auxin action. *Science* **98**:454–455.

BERTRAND, G., and MUTTERMILCH, W. (1907). Sur l'existence d'une tyrosinase dans le son de froment. *Compt. rend.* **144**:1285–1288.

BLAGOVESHCHENSKIĬ, A. V. (1939–40). Utilizing oxidizing systems in the baking of bread from spoiled flour. *Khlebopekarnaya Prom.* No. 4–5:17–19 (1939) and *Khim. Referat. Zhur.* No. 3:126 (1940). (*Chem. Abstracts* 36:2634.)

BLISH, M. J., and BODE, C. E. (1935). Catalase activity in wheat flour. *Cereal Chem.* 12:133–142.

BORNAND, M. (1921). Vegetabile Katalasen und Anwendungen der Katalase in der Nahrungsmittelhygiene. *Lebensm. Hyg.* 12:125–133.

BROWN, A. H., and GODDARD, D. R. (1941). Cytochrome oxidase in wheat embryos. *Am. J. Botany* 28:319–324.

BUNZELL, H. H., and FORBES, M. (1930). A method for testing ropiness of bread. *Cereal Chem.* 7:465–472.

—— and KENYON, M. (1932). On rope control. *Cereal Chem.* 9:161–168.

DIXON, M. (1943). *Manometric Methods.* 2nd ed. Macmillan, New York.

ELION, E. (1942). The action of hydrogen peroxide on wheat flour when used in combination with reducing agents. *Cereal Chem.* 19:594–597.

EULER, H. v., and MALMBERG, M. (1936). Vitamins and catalysts in wheat embryo. *Arkiv Kemi, Mineral. Geol.* B12, No. 14: 6 p. (*Chem. Abstracts* 30: 3860.)

FEASTER, J. F., and CATHCART, W. H. (1941). Value of ascorbic acid from various sources in baking. *Cereal Chem.* 18:201–208.

FERNĀNDEZ, O., and PIZARROSO, A. (1921). El podor catalitico de las harinas. *Anales soc. españ. fís. quím.* 19:265–268.

FRASER, J. G. C., and GFELLER, F. (1936). Inheritance and use of the phenol colour reaction in hard spring wheats. *Sci. Agr.* 17:243–249.

FREILICH, J. (1941). A method for the determination of reducing matter in flour. *Cereal Chem.* 18:129–137.

GELISSEN, H. C. J. H. (1924). Process for bleaching and improving flour and milling products. U. S. Pat. 1,483,546, March 8.

GODDARD, D. R. (1944). Cytochrome C and cytochrome oxidase from wheat germ. *Am. J. Botany* 31:270–276.

HAAS, L. W., and BOHN, R. M. (1934). Bleaching bread dough. U. S. Pats. 1,957,-333/4/5/6/7, May 1.

HAUGE, S. M., and AITKENHEAD, W. (1931). The effect of artificial drying upon the vitamin A content of alfalfa. *J. Biol. Chem.* 93:657–665.

IVANOFF, N. N. (1932). Über die Veränderlichkeit des Fermentgehalts in Samen und Früchten. *Biochem. Z.* 254:71–87.

JØRGENSEN, H. (1935). Über die Natur der Bromatwirkung. *Mühlenlab.* 5:113–125.

KNYAGINICHEV, M. I., and PALILOVA, YU. K. (1940). The activity and quality of catalase in wheats, barleys and genera related to wheat. *Biokhimiya* 5, No. 1:55–64 (in English).

KRETOVICH, V. L., and SOKOLOVA, A. I. (1942). The dehydrogenases of the wheat embryo. *Biokhimiya* 7:232–237.

LAITINEN, H. A., and SULLIVAN, B. (1941). The application of the dropping mercury electrode to the study of oxidation-reduction systems in flour. *Cereal Chem.* 18:60–73.

LIECHTI, P. (1909). Die Prüfung von Mehlen auf Grund ihres Gehaltes an Katalase. *Chem.-Ztg.* 23:1057.

LINDET, L. (1920). L'action de l'eau oxygénée sur les farines. *Compt. rend. acad. agr. France* 6:843.

MARION, F. (1920). Action de l'eau oxygénée sur les farines. *Compt. rend.* 171: 804–806.

MAROTTA, D., and KAMINKA, R. (1922). La decomposizione dell'acqua ossigenata come mezzo di determinazione del grado di abburattamento della farine. *Giorn. chim. ind. applicata* 4:249–251.

MELVILLE, J., and SHATTOCK, H. T. (1938). The action of ascorbic acid as a bread improver. *Cereal Chem.* 15:201–205.

MERL, T., and DAIMER, J. (1921). Studien über Mehlkatalase. *Z. Untersuch. Nahr.-u. Genussm.* 42:273–290.

MICZYŃSKI, K., JR. (1938). Genetische Studien über die Phenolfarbenreaktion bei Weizen. *Pflanzenzücht.* 22:564–587.

MILLER, M. (1909). Beiträge zur chemischen Kenntnis der Weizenmehle. *Z. ges. Getreidew.* 1:194–200, 214–222, 238–244.

NEUENSCHWANDER, N. (1928). Enzymchemische Studien in Weizenmehlen. *Biochem. Z.* 199:445–450.

POTEL, P., and CHAMINADE, R. (1935). Sur le potential d'oxydo-réduction des farines. *Compt. rend.* 200:2215–2217.

PROKOPENKO, N. (1927). The enzyme content of germinating wheat. *Nauch. Agron. Zhur.* 4:346–354.

—— (1928). The enzyme content of germinating wheat. *Deut. landw. Rundschau* 1:824. (*Chem. Abstracts* 22:2765.)

PROKOSHEV, S. M., MARGOLINA, T. I., and BABICHEV, I. A. (1936). The physiological action of hydrogen sulfide on wheat seed. *Bull. Applied Botany, Genetics Plant Breeding* U.S.S.R. *Ser. III*, No. 14, 3–23 (in English).

PROSKURYAKOV, N. I., and PAVLINOVA, O. A. (1940). Ascorbic acid and proteolysis of flour. *Biokhimiya* 5:443–451. (*Chem. Abstracts* 35:4859.)

SHEN, T., and GEDDES, W. F. (1942). Protease activity and reducing matter content of wheat flour doughs in relation to baking behavior. *Cereal Chem.* 19: 609–631.

STERN, R. (1944). Effects on flour doughs of extracts from unfermented and fermented wheat germ. *Cereal Chem.* 21:81–97.

STRAIN, H. H. (1941). Unsaturated fat oxidase: specificity, occurrence and induced oxidations. *J. Am. Chem. Soc.* 63:3542.

SULLIVAN, B., and HOWE, M. (1937). The isolation of glutathione from wheat germ. *J. Am. Chem. Soc.* 59:2742–2743.

——, ——, and SCHMALZ, F. D. (1936). On the presence of glutathione in wheat germ. *Cereal Chem.* 13:665–669.

——, ——, ——, and ASTLEFORD, G. R. (1940). The action of oxidizing and reducing agents on flour. *Cereal Chem.* 17:507–528.

SUMNER, J. B., and DOUNCE, A. L. (1937). Crystalline catalase. *Science* 85: 366–367.

—— and —— (1937a). Crystalline catalase. *J. Biol. Chem.* 121:417–424.

—— and —— (1939). Catalase II. *J. Biol. Chem.* 127:439–447.

—— and SUMNER, R. J. (1940). The coupled oxidation of carotene and fat by carotene oxidase. *J. Biol. Chem.* 134:531–533.

SUMNER, R. J. (1942). Lipid oxidase studies II. The specificity of the enzyme lipoxidase. *J. Biol. Chem.* 146:211–213.

—— (1942a). Lipid oxidase studies III. The relation between carotene oxidation and the enzymic peroxidation of unsaturated fats. *J. Biol. Chem.* 146:215–218.

—— (1943). Lipoid oxidase studies. A method for the determination of lipoxidase activity. *Ind. Eng. Chem., Anal. Ed.* 15:14–15.

TAUBER, H. (1940). Unsaturated fat oxidase. *J. Am. Chem. Soc.* 62:2251.

THUNBERG, T. (1931). Über das Vorkommen einer auf Adenosintriphosphorsäure eingestellten Dehydrogenase in Pflanzensamen. *Lunds Universitets Årsskrift*, N.F., Afd. 2, Bd. 27, Nr. 10, 17 pp.

VAN DER LEE, G. (1932). Process for enhancing the action of per-compounds which are decomposed by catalases upon animal or vegetable products. U. S. Pat. 1,866,412, July 5.

VAN FLEET, D. S. (1942). The development and distribution of the endodermis and an associated oxidase system in monocotyledonous plants. *Am. J. Botany* 29:1–15.

—— (1942a). The significance of oxidation in the endodermis. *Am. J. Botany* 29: 747–755.

VAN FLEET, D. S. (**1943**). Unsaturated fat oxidase: distribution, function and histochemical identification in plant tissues. *J. Am. Chem. Soc.* **65**:740.

VOSS, J. (**1938**). Über sorteneigene Oxydations- und Reduktionsfermente bei *Triticum sativum* L., ihre Verwendbarkeit zur Sortenunterscheidung. *Angew. Botan.* **20**: 265–293, 333–345.

WENDER, N. (**1905**). Die Feinheitsbestimmung der Mehle. *Z. Untersuch. Nahr.-u. Genussm.* **10**: 747–756.

—— and LEWIN, D. (**1904**). Die katalytischen Eigenschaften des Getreides und der Mehle. *Oesterr. Chem.-Ztg.* **7**:173–175.

ZALESSKIĬ, V., and KUKHARKOVA, A. (**1928**). Enzymic oxidation of oxalic acid by higher plants. *Ukrain. Khem. Zhur.* **3**, Part 2, 139–152. (*Chem. Abstracts* **23**: 2991.)

CHAPTER VIII

PROTEASES*

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The splitting of carbon from nitrogen is a process of universal biological importance. Both hydrolytic and oxidative splitting are catalyzed by enzymes. The splitting of ammonia from typical amines seems to be effected by oxidative enzymes at least as far as present examples go. Hydrolytic splitting is probably restricted to the scission of the bond between CO and NH that occurs in acid amides and peptides. That there is no hard and fast differentiation between amide and peptide hydrolysis is shown by the fact that peptide-splitting enzymes frequently act in the capacity of amidases, for instance on such substrates as leucinamide. Neverthless, there is a customary and proper distinction between the *amidases*, which liberate ammonia hydrolytically from arginine, glutamine, etc., and the *proteases*, which are involved in the degradation of proteins and their split-products. Among proteases, the *proteinases*, enzymes that hydrolyze true proteins, are usually distinguished from the *peptidases*, which attack protein decomposition products, namely the various di- and polypeptides resulting from proteinase action. This distinction is of doubtful theoretical value, because so many proteinases are known to hydrolyze peptides that it may be true all of them can. On the other hand, the velocity of the hydrolysis of peptides by proteinases is very small compared with the rate of their action on true proteins, and there is no instance of protein hydrolysis by a (supposedly) pure peptidase. Thus, for practical purposes, the proteolytic enzymes (proteases) may be very conveniently divided into peptidases and proteinases.

Both proteinases and peptidases are doubtless proteins. Many of the former class have been crystallized, and none has so far shown evidence of a prosthetic group. The active portion of a proteinase molecule is probably nothing but a peculiar (and certainly unknown) type of arrangement

* Enzyme Research Laboratory Contribution No. 89.

of the amino acids in that part of the protein. Tyrosine seems to be involved in such an arrangement in pepsin, and cysteine in papain. Only one peptidase (carboxypeptidase) has been crystallized, and this substance has also shown no evidence, except its catalytic property, of being an "extraordinary" crystalline protein. Other peptidases, to be discussed later, act as though they are metal proteins.

The division between proteinases and peptidases is based on practical interpretations of the specificity of the two classes, but an unlimited number of enzyme preparations of each class may be made from the endless list of animals, plants, and microorganisms found in nature. It is probable that no two of these enzymes are exactly alike, for the reason that no two species of living organisms produce exactly the same proteins. In most cases, such differences may well be too minor to cause concern to the enzyme chemist, but it is not surprising that small differences in chemical properties, crystal form, and enzymatic specificity should be found between specimens from different sources of what appears to be the same enzyme. For example, Herriott (1941) crystallized a protein from hog stomach that inhibits the action of crystalline hog pepsin and also of beef pepsin, but not that of crystalline chicken pepsin. In classifying enzymes, it is often difficult to decide how much stress to lay upon occasional differences in specificity of action between different preparations.

It appears to be a general rule in nature for both oxidative and digestive processes to occur in a series of stepwise reactions, thus avoiding an explosive type of change. The catalysts responsible for the individual steps occur usually in groups or systems, which appear to us as mixtures of enzymes. Thus proteinases and peptidases are frequently found together, the latter completing the work of the former. The process of protein digestion in nature is thus brought about by two (or more) groups of enzymes, which appear to work in succession. The separation of the individual members of a group is artificial. It permits the several steps and catalysts of the process to be examined by themselves. But it is not a foregone conclusion that any mixture of the artificially isolated enzymes is the same as the natural mixture from which they were made. The proteins have probably been altered in the process of isolation.

The usual methods of separating enzymes have already been discussed. The separation of proteases involves nothing additional. There are no fixed rules for the separation of enzymes, and usually no single operation succeeds in preparing material pure enough to crystallize. In general, proteinases have been most successfully separated and purified by fractional precipitation with salts, peptidases for the most part by adsorbents of the type originally employed by Willstätter and Waldschmidt-Leitz (1923), including kaolin and various suspensions of aluminum hydroxide.

The present knowledge of proteinases and their substrates is of a totally different type from that now applying to peptidases and peptides: about the enzymes of protein hydrolysis we know very much more than of the peptidases—of their substrates, the proteins, very much less. The peptidases are classified according to the peptides they hydrolyze; the proteinases according to the properties of the enzymes themselves, because they have been obtained in pure form as crystalline proteins.

PROTEINASES

General Considerations

Types of Proteinases. With all due reservations about the future, it appears presently that four general types of proteinases exist, together of course with some borderline examples.

1. Proteinases most active in neutral or slightly alkaline media, thus resembling trypsin. These are frequently called trypsinases or tryptases.

2. Proteinases most active in highly acid media, thus resembling pepsin. They have been referred to as pepsinases, but pepsin from numerous sources appears to be the only common example.

3. Proteinases inactivated by oxidants, and activated by reducing agents such as sulfhydryl and cyanide. These are the papainases. The enzyme proteins in the active (*i.e.*, reduced) form are thought to contain free SH groups. All the known examples of this class are of plant or microbial origin.

4. Proteinases of cellular origin, not requiring reduction to an active state, and with hydrogen-ion optima at weakly acid levels. These enzymes are usually referred to as cathepsins. Cathepsins, being cell proteinases, are endoenzymes as compared with the proteinases of body fluid and plant latices. The term has sometimes been used lately to designate enzymes of animal cells only. There are, however, a few examples of similar enzymes in the plant world.

While the protein-digesting enzymes from many sources are superficially the same, it is obvious that they must be different at least in so far as they reflect species' differences among proteins. Thus it is not surprising that differences in both properties and specificity have been noted and many apparent contradictions occur in the literature. Separating mixtures of enzymes with unclearly demarked specificities offers a problem in that unless the particular specific difference is known it is not evident when such a separation has been successful, or complete. To have blamed trypsin for energetically clotting milk before the discovery of the chymotrypsin mixed

with it is a pardonable error, but nevertheless it resulted in a confused picture of the relation of proteolytic activity to milk clotting, especially during an attempted purification of the one known enzyme.

The Protein Substrate and the Enzyme as Protein. There is no satisfactory chemical definition of a protein. The best is probably that describing a protein as a substance of large molecular size, the greater part of which ultimately yields amino acids on hydrolysis. Knowledge of protein structure is too meager to allow even a satisfactory method of classification. X-ray analysis and the ultracentrifuge have contributed most to what we know about the stereochemistry of the proteins. It appears that they may be divided into two classes, fibrous and nonfibrous proteins. Nonfibrous proteins are crystallizable; fibrous proteins are not. X-ray studies have shown fibrous proteins to consist of long chains which are folded in more or less zigzag fashion making it possible to stretch the substance. The crystalline proteins consist of huge rounded molecules, some spherical and others elongated, but all alike for the same crystallized protein. By virtue of polar groups, strategically situated, protein molecules may attract water, acids, bases, and other suitable ionic material to themselves. They may also attract each other, as indicated by antigen–antibody reactions, such as the combination of diphtheria toxin and antitoxin, and by the formation of enzyme–substrate compounds.

Bergmann and Niemann (1938) have pointed out that, in very many proteins which have been well analyzed, the amino acids occur in such proportions as to indicate definitely recurring patterns of amino acids in the protein chains. It seems likely, therefore, that particular groupings of amino acids constitute a locality in the chain vulnerable to attack by a particular proteinase. Bergmann, in a long series of studies, has attempted to define these localities for several proteinases by observing what synthetic peptides they split most easily. Thus trypsin seems particularly active on lysine and arginine peptides, and in proteins may attack linkages involving those amino acids.*

If the action of a proteinase on a protein is determined by the existence of regularly recurring vulnerable spots, the end-product should be the largest protein fragment that contains no more vulnerable groupings. This

* This is a purely chemical explanation of proteinase action. However, the architectural position of an amino acid in the protein molecule must also be an important factor in determining its vulnerability to enzyme attack in the case of native proteins, whose inner structures are likely to be quite inaccessible to anything so large as another (enzyme) protein molecule. With denatured proteins, the case is different, for these are thought to exist in films, even though the film may be folded. This may be the reason why native proteins as a rule are almost immune to attack by proteinases. None of the work reported above was carried out on native proteins for substrates.

gives great interest to attempts to determine the size of the residual fragments.

The progress of digestion of a protein by a proteinase may be observed by electrophoretic measurements or in the ultracentrifuge. Work with egg albumin being digested by papain (Annetts, 1936) and by pepsin (Tiselius and Eriksson-Quensel, 1939) leads to the conclusion that the enzyme remains with individual protein particles to break them down as far as possible before attacking others. The composition of a digesting mixture consists, therefore, of some protein that is almost unchanged and other that has been broken down to very small fragments, with little or nothing of intermediate size between. Winnick (1944) has studied the partial hydrolysis of casein by pepsin, trypsin, papain, and other proteinases, and concludes from the chemical composition of the products isolated by electrodialysis that the same type of breakdown occurs in these cases. On the other hand, Petermann (1942) has observed (by the centrifuge method) a progressive breakdown of beef serum pseudoglobulin and of horse serum diphtheria antitoxin upon digestion by papain.

Under the influence of heat, alkali, or certain reagents such as urea and guanidine, proteins may suffer a change or series of changes called denaturation. The protein becomes markedly less soluble at its isoelectric point and sulfhydryl groups not previously responsive to tests therefor are detectable after this change. Denaturation is usually regarded as including an unfolding of the molecule probably followed by further configurational changes. No denatured protein has ever been crystallized, but crystalline proteins may undergo denaturation (using the x-ray diffraction pattern as a criterion) without exhibiting any change in their crystal form (Astbury *et al.*, 1935).

Denaturation, at least in its early stages, is frequently reversible, provided some additional change such as precipitation of the denatured protein does not intervene. For this reason enzymes inactivated by heat may, under properly arranged conditions, again become active on cooling. This has been found true of trypsin, chymotrypsin, and bromelin among the proteinases.

Northrop and his associates (see Northrop, 1939) have made extensive studies leading to the conclusion that denaturation of the enzyme protein is accompanied by the disappearance of proteolytic activity. The loss of activity has always paralleled the amount of protein denatured, no matter what means was employed to bring the denaturation about.

Aside from any tendency to reactivation on cooling, the actual stability of various proteinases toward heat is varied. The plant enzymes on the whole are able to withstand higher temperatures. The way to tell whether an enzyme is unharmed at a high temperature, or is inactivated but

recovers its activity later on standing at a lower temperature, is to test its rate of proteolysis at the high temperature. By such an experiment it will be found that papain at 80°C is very active (although gradually destroyed), while trypsin is inactive but recovers activity after a short period in the cold.

Crystallization of Proteinases. There is no definite method for the crystallization of an enzyme. In each case the crystallization of a protein is involved, and every protein must be handled in accordance with its own particular properties. The proteinases are, relatively speaking, simple proteins. Their crystallization does not appear to be complicated by the presence of prosthetic groups attached to the enzyme proteins, such as Warburg has found with several oxidizing enzymes.

In undertaking a crystallization, the general procedure is first to obtain the desired enzyme protein as free as possible from other substances. This usually requires large quantities of material at the start. When one protein represents over half of a mixture of proteins, it is often possible to obtain crystals of it, which may later be used as seed.

Any of the general methods for separating mixtures of proteins may be found suitable as a prelude to their crystallization. The most widely used have been:

1. Precipitation and removal of unwanted material by acidification.

2. Heating to denature any other proteins present that are less stable toward heat than the protein desired.

3. Fractionation by salts, especially ammonium sulfate, and by alcohol, acetone, or dioxane.

4. Fractional electrophoresis.

5. Adsorption on an inorganic material that has been found to adsorb the desired enzyme or undesired impurities with a fair degree of specificity.

6. Specific precipitation of the desired enzyme or of undesired impurities of protein nature by protamines.

These and other methods of separating proteins will be found extremely useful. The order in which they are used generally affects the results. As a rule it is better to use two or more methods successively than one repeatedly.

Actual crystallization of the nearly pure protein has been accomplished by slow precipitation with salts (Northrop, 1939) or with organic solvents such as acetone and dioxane (Sumner, 1926; Sumner and Dounce, 1937, 1939) under conditions found by many trials to be the best for the case in hand. For details the papers of Northrop, of Sumner, and of Warburg (*e.g.*, Warburg and Christian, 1943) should be carefully studied.

Criteria of Purity. Since so many proteinases have been crystallized, the question of whether the crystals represent pure substances becomes of special importance. Two methods are available for deciding whether a material is one protein or a mixture of two or more. The electrophoretic method of Tiselius (1937) depends on showing, for a single protein, that it migrates with a sharp boundary under the influence of an electrical current. When this is the case, the indication is that the molecules are all electrically alike, and it is very probable that they are also chemically alike. Similar observations on the boundary of sedimentation in an ultracentrifuge also constitute good evidence. The boundary is sharply defined when all the protein particles behave alike under the influence of gravity. This is not as good evidence as that furnished by electrical migration, because proteins of very different sizes may settle equally fast, provided they are sufficiently different in shape as well.

A second method for demonstrating whether a crystalline enzyme is a single protein is the solubility method developed by Northrop and Kunitz (1930). This is easier to carry out in most research laboratories. It consists of plotting the activity of the solution of an enzyme protein vs. the total quantity of the protein present. As long as the protein continues to dissolve in the solution, the activity naturally rises regularly therewith. When so much protein has been added that more cannot dissolve completely (salt solutions are usually used to lessen the solubility of the protein), it is obvious that if the protein is pure the addition of more protein cannot increase the activity of the solution. However, if the protein material is a mixture, the solution is not likely to become saturated with both components at the same level, particularly as a solution of one protein frequently increases the solubility of another. The result is, then, that the activity measurements show no abrupt change in slope when the point is reached at which some solid remains undissolved. The method has been extensively used by Northrop and co-workers (see Northrop, 1939). Figure 1 shows a curve obtained with crystalline chymotrypsinogen in which the abrupt change of slope indicates the presence of a single protein.

When the material under investigation has not been crystallized, the question of whether one or more similar enzymes exist therein becomes difficult to answer. A kinetic approach is required, with its inevitable chances of error. Such approaches have been made with enzyme systems other than proteases by many English authors, for instance Stickland (1929).

Irving, Fruton, and Bergmann (1941) have developed special methods of analysis for identifying and classifying enzymes—specially but not necessarily proteolytic enzymes—and for examining the nature of enzymes in mixtures. The methods consist of comparing the specific activities of the

enzymes on several substrates, preferably on substrates of known composition. By this method, Fruton, Irving, and Bergmann (1941) found four distinct proteolytic enzymes in beef spleen.

The determination of "proteolytic quotients" has already led to the detection of similarity of action among a number of enzymes where it would otherwise not have been suspected. The method shows promise of further interesting developments but is beset by the difficulty that each enzyme must be studied under the conditions that produce a monomolecular reaction velocity. A monomolecular reaction velocity may often be obtained experimentally at some particular hydrogen-ion concentration; but this concentration is not necessarily the same for the two enzymes under

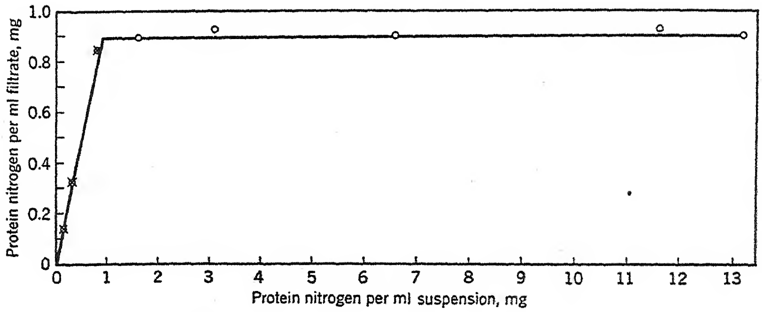


Fig. 1.—Solubility of crystalline chymotrypsinogen in 0.25 saturated ammonium sulfate at 10 °C in the presence of increasing quantities of solid phase (Northrop, 1939).

comparison. Of course no claim is made that such enzymes are the same, but only that their actions are similar, and therefore presumably some details of their structures.

Furthermore, two or more enzymes (already separated) may be compared by determining their specific activities toward two substrates. Bergmann and his co-workers have found a number of instances in which the proteolytic quotient is the same, leading to the conclusion that enzymes from very widely different sources all may attack the same groups in their substrates. Thus, pancreatic trypsin, a proteinase from beef spleen, another from beef kidney, a fourth from hog kidney, and a fifth from crude papain, all had the same proteolytic quotient. In these cases, the ratios of activity toward benzoylarginine amide and benzoyllysine amide were determined. All the enzymes except pancreatic trypsin were activated by cysteine. Bergmann (1942) refers to any two such enzymes as *homospecific* (in comparison with each other), whereas any two enzymes with different proteolytic quotients are called *heterospecific* (again in comparison with

each other). Several carboxypeptidases from different sources were also found to be homospecific with respect to each other.

Thus, Plentl and Page (1944) have attempted to discover whether renin (a proteolytic enzyme from kidney) and pig kidney pepsinase are related enzymes. They have concluded that the two enzymes are not related, because of a difference in proteolytic quotients, although both proteinases give rise to a substance (or substances) capable of raising blood pressure when they act on a serum globulin fraction called "renin-substrate."

Combined Action of Proteinases and Peptidases. Although proteinases and peptidases are both able to liberate amino acids, there is a decided difference in the over-all results achieved in digestion. In practice, the peptidases supplement the action of proteinases, with the result that the protein is ultimately reduced to amino acids to an extent comparable to what occurs on hydrolysis with acids. Calvery (1933) has shown this very neatly by allowing crystalline egg albumin to be completely digested by several enzymes in turn. The results, expressed as amino nitrogen determined in a Van Slyke apparatus, show that simple fractions of the total amino nitrogen present are split off by various enzymes, and that the total, after action by peptidases, represents as complete a breakdown as was obtained by boiling with acid. Table I contains some of the data of Calvery.

TABLE I

FRACTION OF TOTAL AMINO NITROGEN LIBERATED BY EXHAUSTIVE DIGESTION OF CRYSTALLIZED EGG ALBUMIN WITH DIFFERENT PROTEOLYTIC ENZYMES

(From Calvery, 1933)

Sequence of enzymes	Per cent of total amino nitrogen liberated by each enzyme	Sum of the hydrolysis by both enzymes in sequence
I. Pepsin	$^1/_3$	—
Trypsin	0	$^1/_3$
II. Pepsin	$^1/_3$	—
Papain (HCN)	$^1/_3$	$^2/_3$
III. Pepsin	$^1/_3$	—
Aminopolypeptidase	$^1/_3$	$^2/_3$
IV. Pepsin	$^1/_3$	—
"Erepsin" (amino- and dipeptidases)	$^2/_3$	$^3/_3$
V. Acid hydrolysis	$^3/_3$	—

Analytical Determination of Proteinase Activity. Of the available methods, one must be selected to suit the case at hand. The most important consideration is the quantity of proteinase available for the test. When this is small, a delicate method must be used, such as the reduction in viscosity shown by a gelatin solution (Northrop, 1932; Landis and Frey, 1938). When somewhat larger quantities are available, the hemoglobin method of Anson (1938) will be found convenient. Results by these meth-

ods are independent of the concurrent presence of peptidases in the preparation.

The chief advantage of Anson's method over other methods is that the substrate is apparently reproducible. A hemoglobin solution is prepared from defibrinated beef blood by a standard procedure. When frozen, this preparation keeps indefinitely, and small portions can be thawed and used as desired.

A 2 per cent solution of the hemoglobin, containing the proper buffer and denaturing agent, is incubated with the enzyme to be tested. After a short digestion period, usually ten minutes, the remaining protein is precipitated with trichloroacetic acid, and the filtrate is analyzed for reducing substances with the Folin–Ciocalteau phenol reagent. The principal reactants are tyrosine and tryptophan peptides. A unit of proteinase activity is defined by Anson as the quantity of enzyme required to produce a color equivalent to 1 milliequivalent of tyrosine in 1 minute under standard conditions. If the time and temperature of digestion are carefully controlled and if the color developed is measured with a photoelectric colorimeter, this method is extremely sensitive, although not as sensitive as the gelatin viscosity procedure.

In the gelatin method, as used by Northrop (1932), a standard gelatin solution is brought to temperature and mixed with the enzyme to be asayed. The determinations are made at 35.5°C since the viscosity of gelatin remains constant with time at this temperature. The viscosity of this solution is measured at short intervals by means of a viscometer of the Ostwald type. Calculation of the percentage change in specific viscosity may be made from curves of viscosity plotted against time; a change in specific viscosity of 1 per cent per minute is defined as one unit of proteinase activity.

Because the drop in the viscosity of gelatin is a rather complicated function of the time and the enzyme quantity, a set of empirical curves may be prepared with a reference enzyme preparation using various amounts of the enzyme and measuring the viscosity at arbitrarily chosen times. By reference to these curves, the value of an unknown preparation can be expressed in milligrams of the reference enzyme. Balls and Hale (1938) found this procedure very satisfactory for measuring wheat proteinase.

Northrop (1932) has also used the formol titration for measuring proteinase activity. Casein, gelatin, edestin, or other proteins may be used as substrates. The protein and enzyme solutions are mixed and incubated at 35.5° for 20 minutes. Sufficient strong alkali is added to make the solution just pink to phenolphthalein. After the addition of 1 ml of 40 per cent formaldehyde the mixture is titrated with $N/50$ alkali.

Standard curves may be prepared by using different quantities of a

reference enzyme preparation for the same time interval or by determining the amount of digestion with a constant quantity of enzyme for different times. Northrop and Kunitz (1932) obtained the same results either way

TABLE II

SOME BETTER-KNOWN PROTEINASES

Name	Source	Approximate *p*H optimum	Notes	References
Pepsin	Stomach mucosa	1.5–2.0	Pepsinogen activated by pepsin	Northrop (1930)
Trypsin	Pancreas	8–9	Trypsinogen activated by trypsin or enterokinase	Northrop and Kunitz (1932)
Chymotrypsin	Pancreas	8.0	Chymotrypsinogen activated by trypsin	Kunitz and Northrop (1935)
Cathepsin	Glandular tissue, muscle, and brain	4		Anson (1940)
Thrombin	Plasma	6.5	Prothrombin activated by thrombokinase and trypsin	Eagle (1937)
Rennin	Calf stomach mucosa	5	Prorennin activated by acid, about *p*H 5	Hankinson (1943)
Yeast proteinase	Brewers' yeast	5[1]	Activated by hydrogen sulfide	Grassmann and Dyckerhoff (1928a); Grassmann and Schneider (1936)
Bacterial proteinases (four)	Aerobic, anaerobic bacteria	7.0	One activated by cysteine; others not	Maschmann (1939)
Papain	Latex and green papaya	6.5–7.0[1]	Activated by hydrogen sulfide, cysteine, CN, etc.	Balls and Lineweaver (1939)
Bromelin	Pineapple	6.5–7.0	Activated by hydrogen sulfide, cysteine, CN, etc.	Balls, Thompson, and Kies (1941); Greenberg and Winnick (1940)
Ficin	*Ficus*	5.0	Activated by hydrogen sulfide, cysteine, CN, etc.	Walti (1938)
Asclepain *m*	*Asclepias mexicana*	7.5	Activated by hydrogen sulfide, cysteine, CN, etc.	Winnick *et al.* (1940); Carpenter and Lovelace (1943)
Asclepain *s*	*Asclepias speciosa* (milkweed)	—		
Mexicain	*Pileus mexicanus*	—	Activated by hydrogen sulfide, cysteine, CN, etc.	Castañeda *et al.* (1942)
Solanain	*Solanum elaeagnifolium*	7–7.5	Not activated by SH	Greenberg and Winnick (1940); Bodansky (1924)
Squash proteinase	Squash family	—	Not activated by SH	Willstätter *et al.* (1926); Ambros and Harteneck (1929)
Hurain	*Hura crepitans*	8.0[2]	Not activated by SH	Jaffé (1943)
Pinguinain	*Bromelia pinguin*	—	Activated by hydrogen sulfide, cysteine, CN, etc.	Asenjo and Capella de Fernandez (1942)
Proteinase of grain	Wheat, sprouted wheat, barley, malt, etc.	5	Activated by hydrogen sulfide, cysteine, CN, etc.	Balls and Hale (1938); Mounfield (1936, 1938); Olcott, Sapirstein, and Blish (1943)

[1] On casein. The optimum varies with the substrate.
[2] On gelatin.

with crystalline trypsin. Since the amount of digestion in this case was determined by the product of the enzyme concentration and the time, a given value for the product represents a constant amount of digestion, no matter how the enzyme concentration or time was varied. One unit by this method is defined as an increase in amino (or carboxyl)* groups at

* Depending on whether or not the *Zwitterion* theory is the accepted one. According to the classical theory, carboxyl groups were measured by this method.

35.5°C at the rate of 1 milliequivalent per minute in the standard procedure.

There are, of course, other means of determining proteinase activity. Among them are: milk clotting (see page 254), alcohol titration (page 261), and increase in either nonprotein nitrogen or amino nitrogen. They all work well with pure and potent enzyme preparations, but leave much to be desired when the materials tested are weak in proteolytic activity.

Some Well-Known Proteinases. In the following detailed discussion of several well-known proteinases (Table II), the examples are chosen from those we know most about. Proteinases are to be found everywhere that nitrogen is metabolized. The proteinases of rattlesnake and other venoms are apparently very potent. Plant and microorganism proteinases are, however, the cheapest, and therefore the most important industrially. Osage orange contains an active proteolytic enzyme. The method of growing molds on moist bran, and thereafter using the bran as a source of many enzymes is much practiced. Papain has been in the past the cheapest source of proteolytic activity; but recent restrictions on its importation have called attention to the importance of other sources.

Animal Proteinases

Pepsin. Pepsin was the first proteinase to be discovered (Schwann, 1836) and the first to be crystallized (Northrop, 1930). In Figure 2 are

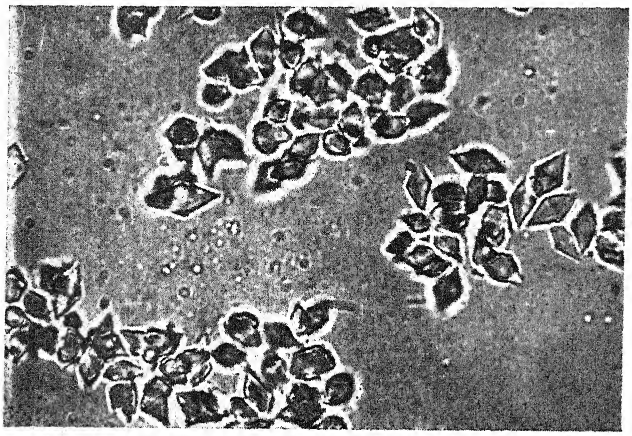


Fig. 2.—Crystalline pepsin.

shown pepsin crystals made by Northrop's procedure. The crystals usually contain an impurity which appears to be a decomposition product of pepsin itself (Northrop, 1939). Accordingly, it is not surprising that pepsin preparations have been made electrophoretically (Tiselius *et al.*, 1938) whose

potency exceeds that of the ordinary crystals. In crystallizing pepsin, Northrop (1931) separated it from another proteolytic enzyme of the stomach, which he called gelatinase because of its specially marked ability to hydrolyze gelatin. This was the first clear-cut example of the separation of similar enzymes by crystallization; the method has since become one of the most reliable for separating enzymes.

For practical purposes, pepsin may be defined as a proteinase active only in highly acid media. Hog pepsin, for instance, is most active at *p*H 1.5–2.0. Similar proteinases from the digestive tracts of several species have been crystallized. These include hog, beef, chicken (Northrop, 1939), and salmon (Norris and Elam, 1940).

In the gastric mucosa, however, pepsin exists as the proenzyme pepsinogen. The conversion of pepsinogen to pepsin is a specific proteolysis, carried out in the acid medium of the stomach, by traces of pepsin. Pepsinogen is converted first to an inactive pepsin inhibitor compound which dissociates to a greater or less extent depending upon the hydrogen-ion concentration of the solution, almost complete dissociation taking place below *p*H 5.0 (Herriott, 1938a). This inhibitor has been crystallized and investigated by Herriott (1941). The peptic inhibitor from hog inhibits beef pepsin also, but not chicken pepsin. Chicken pepsin is also characterized by a moderate stability in alkali (at *p*H 8–9) that would at once destroy hog and beef pepsins. Pepsinogen, on the other hand, is rather stable in alkaline solution (Tauber and Kleiner, 1933), and Herriott (1938) has made use of this property to determine pepsin and pepsinogen in the presence of each other by destroying the pepsin with alkali, activating the remaining pepsinogen by acidification, and finally determining the pepsin so produced. It is necessary to point out, however, that the efficiency of conversion of pepsinogen to active pepsin (and not to pepsin–inhibitor compound) depends on the hydrogen-ion concentration of the conversion mixture; so the conditions in Herriott's method need to be carefully controlled.

Crystalline pepsin protein has been investigated in much detail. It has been discovered thereby that the protein structure may be modified considerably without destroying the proteolytic properties. If pepsin is progressively acetylated by ketene, a series of crystalline acetyl pepsins may be obtained (Herriott and Northrop, 1934). The acetylation of three or four groups in the pepsin molecule reduces the specific activity very little, if at all. The acetylation of between six and eleven groups reduces the activity to about 60 per cent of the original, but the original activity may be regained by cautious saponification with normal sulfuric acid in the cold. When between 20 and 30 groups are acetylated per protein molecule, the resulting product has only 10 per cent of the original activity. The

results have been interpreted as indicating that the first attack of ketene on the protein is made on the ϵ-amino groups of lysine which are thus shown to be nonessential to the proteolytic activity; subsequent acetylation occurs on tyrosine groups that are to be regarded as essential. The cold acid saponification restores some free phenol groups in the protein, which are determinable by the usual Folin procedure (Herriott, 1935).

Iodine inactivates pepsin, and Herriott (1937) has isolated diiodotyrosine from the hydrolytic products of the enzyme so treated. The behavior of nitrous acid is curious. Philpot and Small (1938) found that half of the tyrosine in the enzyme protein reacted with nitrous acid, leaving half of the original activity. Further nitrous acid, however, decreased the residual activity very little. It is evident that some of the tyrosine in pepsin is connected with its enzymatic activity, but this is by no means the whole story. The mode of action of pepsin, as of the other proteinases, is still obscure.

All of the well-investigated proteinases are able to split some peptides slowly, and the sort of peptide split is considered by Bergmann and his collaborators to show the type of substrate and linkage which the enzyme attacks. The action of pepsin apparently represents the hydrolysis of an acidic substrate by an acid enzyme. Thus pepsin (Fruton and Bergmann, 1939) splits tyrosine from carbobenzoxy-*l*-glutamyl-*l*-tyrosine, and from glycyl-*l*-glutamyl-*l*-tyrosine. The acidic nature of tyrosine and of glutamic acid renders the substrate vulnerable to peptic hydrolysis. As a constituent of the substrate the acidic phenylalanine also favors the action of pepsin. Both the sequence of the amino acids and their spatial configuration are of importance as illustrated by the series:

carbobenzoxy-*l*-phenylalanyl-*l*-glutamic acid, split very slowly;
carbobenzoxy-*l*-glutamyl-*l*-phenylalanine, split much faster;
carbobenzoxy-*l*-glutamyl-*d*-phenylalanine, not split.

Rennin (*Chymosin*, *Rennet*). Rennin is considered a proteinase largely by analogy. Its function is to clot milk, and since all proteinases clot milk in some degree, rennin is generally thought of as a proteinase; but evidence that it digests proteins in the ordinary sense is not convincing.

Rennin is found in the stomach mucosa of young animals, particularly calves, where it is gradually replaced by pepsin as the animal grows older. Extracts of the mucosa made with water or dilute acid are used to coagulate milk in the manufacture of cheese.

As pepsin is also a good milk-clotting agent at *p*H 4–5, where its proteolytic properties are not in evidence, it was formerly thought by many workers that rennin and pepsin were the same. Cheese makers, however, have always known that the extract of calf stomach could not be satis-

factorily replaced by that of cow stomach. The pepsin of the latter proteolyzed the casein too much to make good cheese, and also produced a bitter taste. Evidence of the difference between pepsin and rennin now rests on firm ground. For example, Herriott (1941) found that pepsin inhibitor (see page 243) did not affect the milk-clotting power of rennin.

Rennin, like pepsin, occurs as a zymogen or proenzyme; it was prepared first in notable purity by Kleiner and Tauber (1932), who also succeeded in purifying rennin itself by salt fractionation methods until its content of pepsin was very small (Tauber and Kleiner, 1932, 1934).

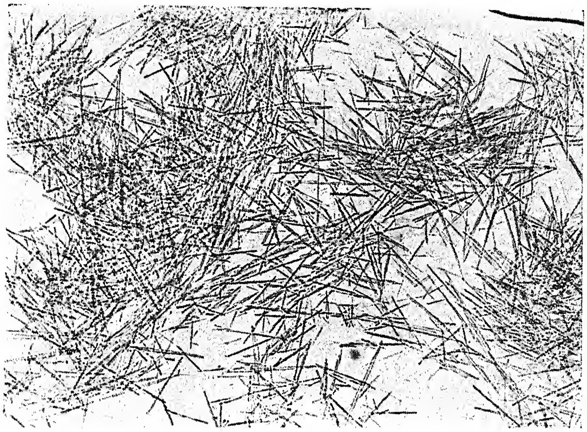


Fig. 3.—Chymotrypsinogen crystals prepared according to the directions of Kunitz and Northrop (1935).

Crystalline rennin has been reported by Hankinson (1943) and by Berridge (1943). The crystalline protein should prove an important tool for studying the chemistry of casein, because, whether proteolytic or not, rennin is quite obviously involved in casein digestion in the young animal until pepsin appears in the stomach to take its place.* A study of the digestion of clotted vs. unclotted casein by trypsin (not chymotrypsin) is clearly required.

The Tryptases. Two zymogens, mother proteins of trypsin and chymotrypsin, are obtained from the pancreas, together with four proteinases derived therefrom. All have been crystallized by Northrop and co-workers (see Northrop, 1939). The mother proteins are trypsinogen

* The digestion of hemoglobin by crystalline rennin (Berridge, 1945) indicates that the enzyme is a protease. The pH optimum (3.7) is quite different from the optimum pH for digestion of hemoglobin by pepsin and is thus not due to contamination by that enzyme.

and chymotrypsinogen. Typical crystals of the latter protein are shown in Figure 3. The zymogens are converted to trypsin and chymotrypsin, respectively (see Fig. 4), but details of the reaction are not definitely known. In the case of trypsinogen, an inert protein as well as active enzyme is formed, depending on the hydrogen-ion concentration of conversion. Chymotrypsin later breaks down slowly in solution to two other enzymes, β- and γ-chymotrypsin. Both chymotrypsinogen and trypsinogen are converted to the respective active enzymes by trypsin, but in neither case by chymotrypsin. Enterokinase, discussed later, also converts trypsinogen to trypsin but has no effect on chymotrypsinogen. Since trypsinogen is converted to trypsin and the trypsin so produced may convert more of the zymogen to more trypsin, the proteolytic conversion of trypsinogen to trypsin is an

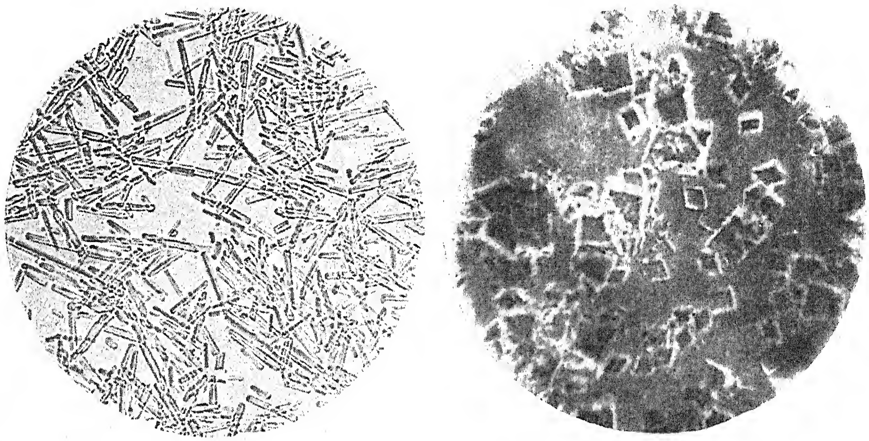


Fig. 4.—Crystalline trypsin (a) and chymotrypsin (b).

autocatalytic process, whereas the proteolysis of chymotrypsinogen to chymotrypsin is not, because the latter change is brought about by trypsin, but not by chymotrypsin.

The autocatalytic conversion of trypsinogen to trypsin is reminiscent in its kinetics of the growth of bacteria in culture media. Kunitz and Northrop (1934) have pointed out that, if a series of tubes is set up containing trypsinogen and the first is inoculated with a loopful of trypsin, the whole tube is soon transformed into trypsin. After a brief incubation, a loopful of the first tube may be placed in the second tube of trypsinogen and so forth with the same result. With no knowledge other than that to be gained from this experiment, the conclusion that the enzyme was alive and growing would be quite in order.

Trypsin and chymotrypsin are proteinases which, tested separately,

are not very different in proteolytic power per milligram of enzyme-protein nitrogen used. Yet not only their crystals but (of more significance) other properties show them to be totally different catalysts. Trypsin clots blood about 700 times more efficiently than chymotrypsin; chymotrypsin clots milk about 80 times better than does trypsin (Northrop, 1939). The action of the two enzymes on casein shows that, while each splits that substrate to about the same extent, the cleavage brought about by the two enzymes occurs at different places. Casein already broken down as far as possible by trypsin may be split further by the addition of chymotrypsin, and vice versa. A mixture of the two enzymes produces about twice the hydrolysis caused by either one alone. But, this is not the case when the two enzymes act on lactoglobulin. Here, some peptide linkages are split in common by both enzymes (Miller, 1939). The first step in the hydrolysis of casein by trypsin or chymotrypsin is accompanied, according to Horwitt (1944), by the appearance of a turbidity, which may be used to measure the initial rate of enzyme action. The action of the two enzymes is different in detail, trypsin, but not chymotrypsin, being inhibited by heparin. Crystalline trypsin inhibitor from pancreas inhibited both enzymes.

When chymotrypsin is crystallized from a solution that has stood some time, the mother liquor contains two more crystallizable proteins that are easily distinguished from each other and from chymotrypsin by their crystal forms and molecular weights, but are indistinguishable as far as known catalytic properties are concerned. They even give the same precipitin reactions with antisera. Kunitz (1938a), who did this work has named the new proteins β- and γ-chymotrypsin. They are presumably formed from chymotrypsin by slow proteolysis, and this idea receives support from the progressively decreasing molecular weights (see Table III).

TABLE III

MOLECULAR WEIGHTS OF TRYPTASES (From Northrop, 1939)

(Osmotic pressure method)

Enzyme	Molecular weight
Trypsin	34,000 ± 1,000
α-Chymotrypsin	40,000
β-Chymotrypsin	30,000
γ-Chymotrypsin	27,000

Enterokinase. Enterokinase occurs in the upper small intestine, and was observed by Schepowalnikow (1899) to activate the precursor of trypsin. Enterokinase may be extracted from dried intestinal mucosa by

dilute ammonia. It is sensitive to heat, but relatively stable to alkali. It has been highly purified by Kunitz (1939) and may be of protein nature. A similar kinase occurs in *Penicillium* (Kunitz, 1938). When enterokinase acts on trypsinogen two compounds may be formed, trypsin protein and an inert protein. The proportion of these two products depends upon the hydrogen-ion concentration of the solution. At *p*H 5–6 and in dilute solutions of trypsinogen, the transformation to trypsin is fairly complete. In more concentrated solutions, the trypsin first formed activates trypsinogen to a measurable extent by the autocatalytic process previously noted. The results of such a complex reaction are hard to interpret. At alkaline levels, where trypsin is proteolytically most active, more of the inert protein is formed than at *p*H 5–6; and more self-activation by trypsin occurs. Kunitz (1939) has succeeded, however, in showing that in acid media enterokinase acts as a catalyst and is therefore an enzyme. If activation is effected in neutral or alkaline media the quantity of trypsin produced by the kinase tends to be smaller, because of the inert protein formed, while that produced by autocatalytic activation of trypsinogen by trypsin (first formed by kinase) becomes larger.

Before the discovery of chymotrypsinogen and its activation to chymotrypsin by trypsin (but not by kinase), chymotrypsin was measured together with trypsin when measurements of activation were made. It is apparent that, under the conditions used by the older workers, the increase in over-all proteolytic activity found after a definite time interval for activation depended most on the amount of trypsin produced at the outset of the experiment by the added kinase. This naturally varied with the amount of kinase employed, and led to results indicating that the greater the dose of kinase, the greater was the total yield of trypsin obtained. Such results (Waldschmidt-Leitz, 1924) were naturally taken to mean that the transformation by kinase was not a catalysis but a compound formation wherein trypsinogen and kinase combined to form an active enzyme complex. This complex is referred to in much of the older literature as "trypsin–kinase."

The tryptic enzymes, besides hydrolyzing proteins, are able to split certain peptides slowly. Amino acids are a regular product of tryptic digestion. Bergmann, Fruton, and Pollok (1939) have studied the specificity of trypsin on synthetic substrates; Bergmann and Fruton (1937a) have studied that of chymotrypsin.

Both trypsin and chymotrypsin are active amidases. Ammonia is split from a number of substituted amino acid and peptide amides, sometimes with surprising rapidity. Thus, hippuryllysine amide is hydrolyzed so fast that with ordinary doses of enzyme the reaction is completed almost instantaneously (Hofmann and Bergmann, 1939). The splitting of

benzoylglycyllysine amide, benzoyllysine amide, and benzoylglycylarginine amide follows first-order kinetics with the reaction constant proportional to the enzyme quantity. Ammonia is also split from unsubstituted peptide amides by chymotrypsin. Among such substrates are carbobenzoxyglycyltyrosine amide and glycyltyrosylglycine amide.

One of the rare examples of experimental synthesis by a proteolytic enzyme was demonstrated by Bergmann and Fruton (1938) with chymotrypsin. Benzoyltyrosine and glycine anilide were coupled to form benzoyltyrosylglycine anilide.

The tryptic enzymes are more stable than most others toward rather vigorous treatment. They are not particularly sensitive toward either acid or alkali; in fact, solutions of trypsin keep best at about *p*H 5. Trypsin may be heated very briefly as high as 100°C, provided the solution is about *p*H 2. Immediately after heating there is no activity, but on standing a short time the enzyme protein reverts completely to the active form. This method was used by Northrop and Kunitz (1932) for removing inert protein in order to crystallize trypsin. In a later paper, Kunitz and Northrop (1936) describe a preparation of crystalline trypsin which does not require this heat treatment.

Trypsin is inhibited by sulfhydryl compounds, but not by cyanide. While trypsin is a potent proteolytic enzyme, its action on proteins in general appears to be greatly aided by their prior denaturation through other causes.

Trypsin is the only known enzyme to possess the peculiar property of transforming proenzymes other than its own to their respective active derivatives. Thus, not only chymotrypsin but also prothrombin and procarboxypeptidase are made active by trypsin. The activation of these proenzymes must require a peculiar and special type of specificity. There is no reason to suppose that such proenzyme substrates are denatured, nor is there any definite proof that the changes produced in the zymogens are actually proteolytic in nature, although they probably are. A physiological function of trypsin, more important than its action as a digestive enzyme, may well reside in its ability to activate many proenzymes.

Cathepsin. Catheptic proteinases occur quite generally throughout animal tissues, but particularly in gland (Willstätter and Bamann, 1929), muscle (Lineweaver, see Balls, 1938), and brain tissue (Edlbacher *et al.*, 1934). Their hydrogen-ion optimum is in the neighborhood of *p*H 4, and they are the proteinases responsible for the autolysis of meat and similar products. Cathepsin from beef spleen has been considerably purified by Anson (1940). It appears to be a rather less active proteinase than the digestive enzymes and also a less potent milk clotter. This impression may be due, however, to the difficulty of removing contaminants.

Formerly cathepsin was thought to be the animal counterpart of papain, in that it was held to be activated by reducing agents, chiefly the sulfhydryl-containing constituents of the tissues. This theory gave a convenient explanation of the connection between sulfhydryl metabolism, oxidation-reduction reactions, and protein metabolism in animal tissues. It is by no means discarded today, Bersin (1935) being among its proponents. While there is little doubt that a connection between sulfhydryl and protein metabolism really exists in animals, it now seems doubtful if the catheptic proteinase is concerned therein. Older data showing the activation of cathepsin by hydrogen sulfide are contradicted by the behavior of Anson's purified enzyme. This preparation is affected neither by reducing agents nor by iodoacetate or iodoacetamide, which are reagents capable of destroying the sulfhydryl groups of the papainases. These conclusions are based on Anson's method (1938) for measuring proteolytic activity (page 240). When the proteolytic activity of a crude preparation is measured by formol or alcoholic titration, it is only during the first few minutes of action that the observed effect is due to the proteinase; thereafter peptidases break down the already partly digested protein. Anson's measurements show that, while catheptic proteinase is not affected by hydrogen sulfide, it is accompanied by a peptidase that is. Such a peptidase was described in spleen and liver by Waldschmidt-Leitz, Schäffner, Bek, and Blum (1930), as responsible for the hydrolysis of benzoyldiglycine and other acid peptides, and referred to as a carboxypolypeptidase.

While considering the possible effect of sulfhydryl groups on protein digestion and synthesis, it may be mentioned that the action of SH on the substrate as well as on the enzyme should be taken into account. A reduction of the substrate protein may result in making certain proteins digestible. The mode of action of keratin digestion in the clothes moth is an example. Keratins have been almost unanimously regarded as unattacked by proteinases. Yet the clothes moth and other insects have long been known to utilize keratin nitrogen. Linderstrøm-Lang and Duspiva (1935) found that the digestive tract of such insects excreted sulfhydryl compounds, probably of polypeptide structure. Their effect on the cystine-rich keratins (see also Goddard and Michaelis, 1934) was to reduce the protein disulfide to sulfhydryl. In the moth, such reduced proteins are digested by a proteinase akin to trypsin, though differing therefrom in not being easily inhibited by thiol compounds.

Thrombin. Judged from the literature, the coagulation of blood is one of the most complex phenomena of nature. No attempt will be made to discuss it in detail. It involves at least four proteins and an activator of the kinase type, or else an inhibitor of the clotting process, as well as calcium and certain factors derived from the wounded tissues. The actual

formation of the clot is due to the change of the soluble protein fibrinogen of the plasma to the insoluble fibrous protein, fibrin. This change is brought about by an enzyme called thrombin. Thrombin, like rennin, is regarded as a proteinase, not because there is any clear proof of its proteolytic activity, but because it is difficult to see what else it can be (Eagle, 1937). Recently, however, Chargaff and Bendich (1943) have regarded thrombin as an enzyme of oxidation.

Thrombin occurs in blood plasma as a zymogen, prothrombin. Prothrombin and thrombin have been partially purified. Whatever the action of thrombin on fibrinogen, the change of prothrombin to thrombin is apparently a proteolysis, for Eagle and Harris (1937) have shown that it may be brought about by trypsin. The blood-clotting power of trypsin is therefore an indirect effect, consisting in the production of the enzyme that forms the clot. Pure trypsin has no clotting action on fibrinogen. Papain, which is also an active blood clotting agent, acts differently. Papain has no effect in transforming prothrombin to thrombin, but is able to change fibrinogen to fibrin and thus clot blood directly. Of course this could be either a proteolysis or an oxidation-reduction process. Both trypsin and papain have been used in surgery to stop hemorrhage. The natural activation of prothrombin to thrombin has long been regarded as brought about by a kinase, thrombokinase, whose source is (in part at least) the wounded tissue. Eagle and Harris (1937) regard thrombokinase as possibly a proteolytic substance, similar in action to the also possibly proteolytic enterokinase. An alternative view is held by Dyckerhoff (1940), who regards thrombin as already present in the blood but inactive because of the effect of an inhibitor also present. According to this scheme the inhibitor is removed by thrombokinase.

Plant Proteinases

Almost all the well-known proteinases of plant origin are enzymes of the papain type, so-called papainases. The papainases are proteinases that may be reversibly activated and inactivated by many reducing and oxidizing agents, respectively. Among the most suitable activating agents are hydrogen sulfide, cysteine, soluble cyanides, and sulfites. Hydrogen peroxide, iodine, ferricyanide, and even air cause inactivation. Traces of heavy metals are probably concerned as catalysts of the inactivation, at least by air. In the presence of alkaline cyanides, all the papainases give a positive test for sulfhydryl, but the nitroprusside test for sulfhydryl groups disappears together with the activity on treatment with an excess of an oxidizing agent. No enzyme of animal origin has yet been definitely proved to be a papainase. The only plant enzymes of this type available

commercially at the present time are papain and ficin. The former is used in industry, chiefly as a clarifying agent for beer, while the latter is a pharmaceutical preparation.

A few plant proteinases appear not to belong to the papain group. Willstätter, Grassmann, and Ambros (1926) found that the proteinase in the press juice of the squash was not activated by hydrogen sulfide. The insect-digesting enzyme of the pitcher plant is akin to trypsin in that it acts best in slightly alkaline media (Hepburn and Jones, 1927). A proteinase occurring in droplets on the flowers of *Drosera* (sundew) has been studied by Holter and Linderstrøm-Lang (1933) and found to resemble pepsin somewhat. Its optimum hydrogen-ion concentration is about pH 3, which is not as low as that for pepsin but is nevertheless unusually acid.

Papain. Crude papain is naturally active, and occurs in the latex of *Carica papaya* together with an activator that contains sulfhydryl groups, and among whose constituents Basu and Nath (1936) identified glutathione. The activation of papain with hydrogen cyanide was first observed by Vines (1903) and investigated by Mendel and Blood (1910). Most reducing agents that are not rapidly destructive of proteins activate the oxidized form of the enzyme. Hydrogen sulfide, cyanides, sulfites, and cysteine are most commonly used. Activation, like most reductions, is more rapid in alkaline solutions. The extraordinary resistance of papain to alkali makes it possible to treat the enzyme protein with 2 M potassium cyanide for several minutes at room temperature, thus activating it completely without causing appreciable loss of protein.

Crude papain gives a positive test for sulfhydryl with alkaline sodium nitroprusside. The test disappears, together with the activity, on oxidation. These facts led Bersin and Logemann (1933) to propose that the activity of the enzyme depends upon the presence of reversibly oxidizable and reducible sulfhydryl groups. Previously, Krebs (1930) had claimed that the action of sulfide, cyanide, etc., consisted in the removal of otherwise inhibitory heavy metal ions; but this explanation seems hardly sufficient to account for the inactivating effects of air and iodoacetate.

Fruton and Bergmann (1940), however, believe that oxidation-reduction phenomena are not responsible for the activation of papain; but rather that, in building the active enzyme, cyanide forms with papain a reversibly dissociable addition compound after the fashion of trypsin–kinase. This conclusion was based chiefly on the observation that activated papain became inactive when the activator was separated from the protein by dialysis or precipitation. However, while studying the behavior of ficin, Winnick, Cone, and Greenberg (1944) found that no inactivation occurs if the activator is removed in the absence of air. They conclude that the coenzyme function attributed to cyanide, cysteine, and other activat-

ors actually consists in the protection of the enzyme against inactivation by oxidation or by combination with heavy metals.

An interesting theory of the mode of action of the sulfhydryl enzymes has been proposed by Bersin (1935). One or more of the constituent amino groups of the enzyme protein are thought to be activated by the nearness of sulfhydryl groups, to the extent that an interaction between the enzyme and a peptide bond of the substrate takes place, thus breaking the bond in two steps:

1. $—CO—NH— + NH_2—enz \rightleftharpoons —CO—NH—enz + NH_2—$

2. $—CO—NH—enz + H_2O \rightleftharpoons —COOH + NH_2—enz$

As evidence of the likelihood of such a reaction, there is cited the ease with which Greenstein (1937) hydrolyzed the sulfhydryl-containing anhydrocysteinylcysteine to cysteinylcysteine as compared with the difficult hydrolysis of the otherwise similar anhydroalanylalanine.

The action of papain (plus cyanide) on crystallized egg albumin has been studied with the ultracentrifuge by Annetts (1936). The digestion was made at *p*H 5.0 and 40°C. The breakdown of the protein was observed to occur in at least two steps: a change in all of the molecules, probably a loosening of internal bonds, but productive of particles almost as large as those of the original protein; a second change consisting in the gradual scission of particles of the size of small peptides from these modified molecules. Both fractions are heterogeneous. The picture during the course of digestion is that of a mixture of very large and very small fragments, with little or nothing in between.

Because of its susceptibility to inactivation through oxidation, substrates like hemoglobin reduce somewhat the activity of papain during digestion, particularly in the presence of oxygen (Anson, 1938; Lineweaver and Schwimmer, 1941). The addition of a small amount of cyanide or other activator is necessary in such cases for the stabilization of the activity during an assay. Conversely, it is of great practical interest that papain may become activated by sulfhydryl groups present in the substrate (Purr, 1935) or liberated therefrom during the course of proteolysis (Gottschall, 1944). With a substrate containing much "buried" sulfhydryl, an almost inactive papain becomes greatly activated during the course of digestion. When dealing with papain, which is usually only partly active, it is of importance to consider the composition of the protein to be acted upon. In some cases an added activator is necessary; in others, an almost inactive enzyme preparation will be very effective.

The assay of papain therefore depends greatly on the conditions of the analysis. Previous activation, unless specifically objected to, is quite

advisable. A convenient method of assay has been described by Balls, Swenson, and Stuart (1935), who used casein and determined its digestion by the alcohol titration of Willstätter and Waldschmidt-Leitz (1921). However, there is no reason to suppose that the milk-clotting ability of a crude preparation is not a measure of its proteolytic power. Balls and Hoover (1937) have described a milk-clotting test which is easier and faster than determinations of proteolytic action. A suspension of milk powder (Klim) in acetate buffer is used as the substrate. To 10 ml of this milk at 40°C 1 ml of enzyme preparation (previously activated) is added, and the time required for clotting is observed. That quantity of enzyme

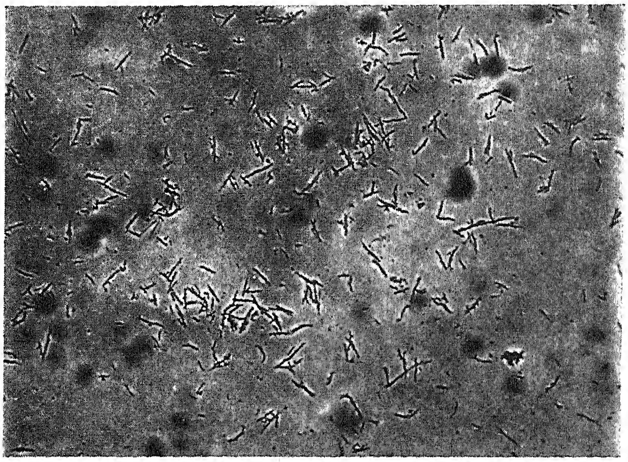


Fig. 5.—Crystalline papain (Balls and Lineweaver, 1939).

which clots the milk in one minute is taken as a unit. For practical purposes the enzyme quantity may be regarded as inversely proportional to the clotting time.

Crystalline Papain and Chymopapain. Balls and Lineweaver (1939) have described a method for crystallizing papain (Fig. 5). Jansen and Balls (1941) have prepared crystalline chymopapain. In both cases, mixed crystals of active and inactive enzyme were obtained. The molecular weight of papain is 27,000 to 30,000, that of chymopapain about 45,000. The protein-digesting power of the latter enzyme, per milligram of protein nitrogen, is half that of the former, but its milk-clotting power is the same. Thus, the ratio of milk-clotting to protein-digesting capacity is double for chymopapain. In crude papaya latex there is probably a third proteinase more proteolytic than papain and possibly a fourth more reninlike than chymopapain.

Crystalline papain, like the prolamines, is soluble in 70 per cent alcohol. Like other proteinases, it splits certain selected peptides and also hippurylamide. The rates are discouragingly slow compared with the rate at which true proteins are split.

Strong solutions of active crystals of papain give no nitroprusside test for free sulfhydryl groups unless some denaturing substance such as urea has been added. Reactive sulfhydryl groups are uncovered by this procedure in papain, as also in egg albumin and other proteins (Anson, 1941). The activity of papain, as shown by Lineweaver and Hoover (1941), is changed little if any by treatment with urea, so that the "uncovering" of the sulfhydryl groups does not seriously affect the essential enzymatic configuration. Balls and Lineweaver (1939a) have shown that such sulfhydryl groups in papain may be titrated by iodine in urea solution, and may also be destroyed in the original protein by iodoacetate, with consequent loss of proteolytic activity. By comparing the loss of activity to the iodoacetate used and the sulfhydryl groups remaining, it was found that one molecule contained about eight sulfhydryl groups, one of which was essential to the activity of the enzyme.

Ficin. Ficin is a proteinase of the papain type found in the latex of plants of the fig family. Such latices have been used in certain localities for many years to clot milk for cheese making, and also for their medicinal properties. *In vitro*, dilute solutions of either crude or purified ficin kill and rapidly disintegrate the bodies of certain species of *Ascaris*, a property due to the presence of the proteinase, and exhibited also by solutions of papain and bromelin. The value of these enzymes as anthelmintics, however, probably depends on whether sufficient quantities escape destruction in the stomach, because the worms are found in the intestine.

Ficin was the first papainase to be crystallized (Walti, 1938). It resembles papain and bromelin in its behavior toward oxidizing and reducing agents.

Bromelin. Bromelin, the proteolytic enzyme of the pineapple family, is found in large quantities in both green and ripe pineapples and also in the press juice of the leaves and stems (Balls, Thompson, and Kies, 1941). The enzyme resembles papain in many ways, though differing in detailed behavior. It is much less stable than papain in alkaline solution. Bromelin has never been obtained in a highly purified state and is probably a mixture of similar proteinases. In crude preparations, the proteolytic activity is not as resistant as papain to heat, but nevertheless bromelin may be recovered in the canning factory from the press juice of the peelings and stalks of pineapples after exposure to about 55°C. The protein fraction of the press juice is easily precipitated by alcohol or ammonium sulfate and yields a crude enzyme preparation, which shows promise of eventually be-

ing a commercially valuable product. The faintly fruity odor is in pleasant contrast to that of crude papain.

Bromelin like papain occurs in fully active form in the plant. Oxidized preparations may be reactivated by reducing agents, apparently in the same way as papain. Bromelin may be assayed by measuring either proteolysis or milk-clotting power. The denaturation of crude bromelin preparations by heat is to a considerable extent reversible on later cooling. The same is true of denaturation caused by alkali. The hydrogen-ion concentration at which the regeneration is carried out affects the extent thereof very considerably (Balls, Thompson, and Kies, 1941).

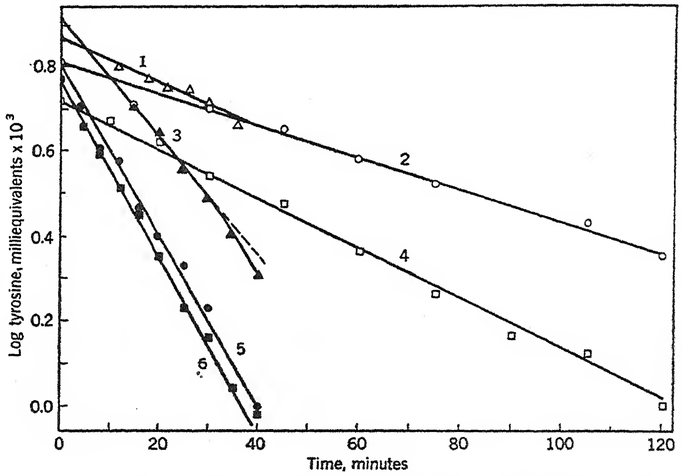


Fig. 6.—Rates of heat inactivation of plant proteinases (Greenberg and Winnick, 1940). Curve 1, papain at 75°; 2, asclepain *m* at 60°; 3, bromelin at 60°; 4, solanain at 75°; 5, asclepain *m* at 65°; 6, solanain at 80°.

Greenberg and Winnick (1940) have made a detailed study of the kinetics of the action of bromelin and several other plant proteinases. Figure 6 shows the rates of heat inactivation of these enzymes. Bromelin, papain, asclepain *m* and solanain follow a first-order reaction course, whereas the heat inactivation of asclepain *s* is apparently a second-order reaction. Although the heat inactivation curves of asclepain *m* and *s* are dissimilar, other evidence points to their being very similar proteins. All of these enzymes appear to differ markedly from solanain in that the last is not activated by reducing substances.

Evaluation of the Michaelis constants for the five plant proteinases —papain, bromelin, asclepain *m*, asclepain *s*, and solanain—indicated that,

in every case, the enzyme substrate intermediary compound consisted of one molecule each of enzyme and protein.

Proteinases of Grains. The papain type of proteinase is apparently widespread in the seeds of grains, but is not the only type present. Thus, Balls and Hale (1938) have identified a proteinase in bran and wheat germ, and also in wheat flour, as being activated by cysteine and hydrogen sulfide. The amounts present are small enough to require delicate methods of measurement such as those based on reduction in the viscosity of gelatin solutions. On the other hand, Olcott, Sapirstein, and Blish (1943) have described a protein-disaggregating enzyme in wheat gluten whose action is

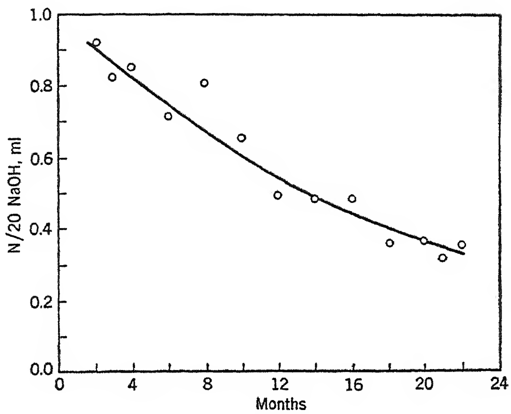


Fig. 7.—Effect of aging of wheat seeds on proteolytic activity. Substrate, 2 per cent edestin in $M/7$ acetate buffer, pH 4.1. Temperature, 40°C. Enzyme, 2:7 dilution. Formaldehyde titration of 4-hour samples. Titers expressed as ml $N/20$ sodium hydroxide per 5 ml sample in excess of a control solution without edestin (Mounfield, 1936).

not increased by sulfides or cysteine, and seems therefore to be distinct from the proteinase in wheat described by Balls and Hale (1938).

The quantity of proteolytic enzymes in grain increases tremendously during sprouting. The proteinase in malt is thus easily demonstrable, and of commercial importance in the manufacture of beer, if the latter is to be made "chillproof" without the addition of proteinases from outside sources. The development of proteinase in wheat seeds during sprouting has been studied by Mounfield (1936, 1938). The enzyme was activated by cyanide, showed an optimum pH of 5.1 on gelatin, and otherwise appeared to be a papainase. The ability to develop proteinase on sprouting decreases with the age of the wheat, as is shown in Figure 7 by data taken from Mounfield (1936a). Here the power to develop proteinase on germination (without regard for whether the wheat germinated or not) dwindled in two years' dry

storage at room temperature to about one-third of the initial value. The author considers this power to be a measure of the vitality of the seeds.

Proteinases of Microorganisms

There is little to be said of microorganismic proteinases, except that they are doubtless legion. Many, like the proteinase of bakers' yeast (Grassmann and Dyckerhoff, 1928a), belong to the papainase group, activatable by cysteine, sulfides, and other reducing agents. The proteinases of several bacteria have been extensively purified and carefully studied by Maschmann (1939, 1940). Each species of bacteria appears able to excrete several distinct enzymes.

From media containing very young cultures of both aerobic and anaerobic bacteria, Maschmann (1939) has obtained four distinct proteinases, each free from the others. These enzymes are all most active at about *p*H 7. Three of them are fully active aerobically and are not activated by cysteine; the other is an anaerobic proteinase, which requires cysteine *in vitro*.

The method of preparation was in general to filter the bacteria from the culture and precipitate the enzyme protein from the latter with methyl alcohol, acetone, or ammonium sulfate. Further purification was obtained by dialysis against mildly alkaline buffer.

1. From 12-hour cultures of *Bacillus pyocyaneus*, *B. prodigiosus*, and other aerobes, a proteinase of wide specificity was concentrated about 1000 times. The enzyme split a very wide selection of substrates, including clupein and egg albumin, and required no cysteine for maximum activity.

2. From 3- to 4-hour cultures of gas-forming anaerobes (Welsh-Fraenkel bacillus, *B. histolyticus*, and others), a proteinase of very narrow specificity was concentrated about 2000 times. Of the many substrates tried it acted only on gelatin and gluten. It is an aerobic enzyme, requiring no cysteine. In this characteristic it recalls the gluten-digesting enzyme in flour, described by Olcott *et al.* (1943).

3. From the above anaerobes and also from *B. botulinus*, a proteinase of wide specificity (when impure) was obtained; it was activatable by sulfhydryl compounds and cyanides. It also required some of the boiled culture filtrate for the development of full activity.

4. From anaerobic cultures of *B. sporogenes*, another proteinase requiring no activator has been obtained. It appears to differ from enzyme No. 1 described above only in minor details of specificity.

Proteinases of bacterial origin may be greatly developed in a given culture by the method used to grow it. When in the possession of commercial concerns the details of such methods are generally held secret. Aeration or the lack thereof appears to play a significant role. Highly proteo-

lytic bacillary bodies of the *B. subtilis* type find occasional use in industry, in competition with papain and similar enzymes.

PEPTIDASES

General Considerations

Types of Peptidases. Synthetic peptides of known composition are available for the study of peptidase action. This has led to an enormous body of data on the specificity of peptidases which would be very valuable if we knew more about the enzymes themselves. Carboxypeptidase has been crystallized by Anson (1937a) and yeast polypeptidase has been isolated by Johnson (1941) as a homogeneous protein. Few if any other peptidases have been obtained in a state of purity that is demonstrably satisfactory. Most statements on peptidases must therefore be taken as referring to a type or group of substances rather than to a chemical individual. There are obviously several types of peptidases whose representatives are sufficiently alike to be considered here together (Table IV). For example, a preparation containing dipeptidase may be made from several sources, such as intestinal mucosa and brewers' yeast. There is some evidence that the enzyme is different when prepared from different sources, because the hydrogen-ion optima, specificity of action on some (but not on all) synthetic substrates and other properties may differ. This evidence is not conclusive, however. The observed differences may be due to various impurities accompanying the enzyme. For the present, it is best in considering the properties of a peptidase to keep in mind not only its type but also its source and method of preparation.

To some extent the specificity of a peptidase may be expressed in terms of the length of the peptide chain that is split by the enzyme. An enzyme is a *dipeptidase* if it splits dipeptides only. If it cannot split dipeptides, but attacks longer chains, it is a *polypeptidase.* The nature and configuration of the constituent amino acids in the chain are also determining factors. The peptides formed naturally from proteins during digestion carry a free amino group at one end of the peptide chain (unless the chain begins with proline or hydroxyproline) and a free carboxyl group at the other. If the presence of the free amino group is necessary for the action of the enzyme, as demonstrated on artificial substrates, it is called an *aminopeptidase.* If the free carboxyl group is necessary, the enzyme is a *carboxypeptidase.* In either case, it is this particular amino acid that is split off by the enzyme. If the residual peptide chain, now shorter by one amino acid, continues to possess the required configuration, the process of attack may be repeated, resulting in the removal of another amino acid. When the peptide chain starts with proline, a special peptidase, *prolylpeptidase,* is re-

TABLE IV

SOME BETTER-KNOWN PEPTIDASES

Name	Source	Example of substrate	Approximate pH optimum	Notes	Reference
Dipeptidase	Small intestine	Leucylglycine	7.8	Inhibited by hydrogen sulfide	Waldschmidt-Leitz *et al.* (1929); Bergmann *et al.* (1935)
	Sprouted wheat	Leucylglycine	7.3–7.9	Activated by hydrogen cyanide	Mounfield (1936, 1938)
	Barley malt	Alanylglycine	7.8	—	Linderstrøm-Lang and Sato (1929)
	Brewers' yeast	Alanylglycine	7.8	—	Grassmann (1929); Grassmann and Klenk (1929)
Leucylpeptidase	Small intestine	Leucyl di- or polypeptides	7–7.5	Activated by magnesium	Johnson *et al.* (1936); Linderstrøm - Lang (1929, 1930)
	Cabbage	Leucyl di- or polypeptides	8.0	Activated by magnesium and manganese	Berger and Johnson (1939, 1939a)
	Barley malt	Leucyl di- or polypeptides	8.6	Activated by magnesium and manganese	Linderstrøm-Lang and Sato (1929); Berger and Johnson (1939a)
	Muscle	Leucyl di- or polypeptides	—	Activated by manganese	Schwimmer (1944)
	Serum	Leucyl di- or polypeptides	—	Activated by magnesium, manganese, and cobalt	Maschmann (1940a,b)
Aminopolypeptidase	Small intestine	Alanyl- and leucyldiglycine	7.2	—	Waldschmidt - Leitz, Balls, and Waldschmidt-Graser (1929)
	Brewers' yeast	Alanyl- and leucyldiglycine	7.0–7.9	—	Grassmann and Dyckerhoff (1928a); Johnson (1941)
Carboxypeptidase	Pancreas	Leucylglycyltyrosine Chloroacetyltyrosine	7.4	Exists also as a proenzyme	Waldschmidt-Leitz and Purr (1929); Anson (1937a); Hofmann and Bergmann (1940)
Catheptic peptidase	Spleen	Benzoyldiglycine	4.2	Two components, one activated by cysteine and one by ascorbic acid	Waldschmidt-Leitz *et al.* (1930); Anson (1937); Fruton, Irving, and Bergmann (1941)
Bacterial peptidases	*B. histolyticus*, etc. *B. prodigiosus* and other aerobes	Leucylglycine and leucyldiglycine	7.8	Mixtures. Activated by iron, manganese, and magnesium after cysteine	Maschmann (1939, 1939a,b, 1940)
	Proteus vulgaris	Leucylglycine and leucyldiglycine	—	Activated by manganese and magnesium	Berger and Johnson (1939)
Prolylpeptidase	Small intestine	Prolylglycine	—	—	Grassmann, Dyckerhoff, and Schoenebeck (1929)
Prolidase	Small intestine	Glycylproline	—	—	Bergmann and Fruton (1937)
		Glycylhydroxyproline	8.0	Activated by manganese	Smith and Bergmann (1944)

quired to separate this amino acid. Peptidases are also widespread in nature that require (or at least greatly prefer) peptide chains which start with leucine. These enzymes are *leucylpeptidases*.

In all the preceding cases, the amino acids separated from the chain have been held by peptide linkages, —CO—NH—; but, if one of the in-

terior amino acids is proline, one bond must possess the configuration, —CO—N(proline). This, at least in some cases, requires a special enzyme, *prolidase*.

The foregoing scheme of describing peptidases tells something of the substrates they act upon, but it does not tell all. It should not be assumed because a peptide carries an initial amino group or a final carboxyl that it must *ipso facto* be split by an amino- or carboxypeptidase, respectively. Each type of peptidase requires substrates that possess other qualifications as well, some of which are discussed later.

It is obvious that these specifications may overlap. A leucylpeptidase is an aminopeptidase with a restricted specificity. It is also apparent that some peptides may be attacked by two enzymes at once. This has been observed, for example, with leucylglycyltyrosine (Waldschmidt-Leitz and Balls, 1930). Aminopolypeptidase removes leucine and, being a polypeptidase, can go no farther. Carboxypeptidase splits off the tyrosine, but can also go no farther because it appears to split only highly acid substrates. The two enzymes together, however, reduce the tripeptide to its component amino acids.

Analytical Methods. The splitting of peptides may be accurately followed by measuring the amount of free amino nitrogen or carboxyl groups newly formed during the hydrolysis. The customary analytical methods are well known and entirely satisfactory. The Van Slyke (1913) method for amino nitrogen is probably the most accurate procedure but does not measure a digestion that uncovers a proline residue. Neither ammonium salts nor urea may be present in considerable quantities. The formol titration of Sørensen (see Northrop and Kunitz, 1932) and the alcohol titration method of Willstätter and Waldschmidt-Leitz (1921) do not have this drawback.

Titration in alcohol is probably the simplest process and is generally accurate enough. A test sample of the digestion mixture is diluted with enough alcohol to bring the concentration of the latter to 88 to 90 per cent by volume and titrated with alcoholic potassium hydroxide to the end-point of a very alkaline indicator, such as thymolphthalein. The alcohol shifts the end-point of the indicator still farther on the alkaline side. Small quantities of free ammonia have no influence in this titration, and the reagents may be standardized by titrating ammonium chloride in the same way. The chief source of error is due to the formation of precipitates by the alcohol. In the presence of much phosphate the end-points are also unsatisfactory. If the method is to be used at all under these circumstances, it is best to start the titration before adding the bulk of the alcohol, which is then added near the end of the titration.

Formol titration has been adopted by several workers for the deter-

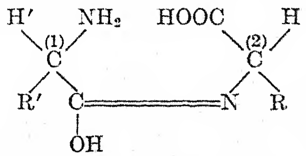
mination of peptidase activity. The enzyme–peptide mixture is titrated with dilute sodium hydroxide before and after digestion. The titrations are carried out in approximately 5 per cent formaldehyde and the end-point determined electrometrically or with phenolphthalein as the indicator (Anson, 1937b; Schwimmer, 1944).

Peptidases *Not* Found to Be Activated by Sulfhydryl or by Metals

Dipeptidase. This enzyme occurs in brewers' yeast and is one of the peptidases of the intestinal "erepsin," formerly considered to be a single enzyme. Dipeptidase, or an enzyme very like it, is widespread and has been reported in malt, milk, pancreas, and leucocytes. Its presence is commonly demonstrated by the hydrolysis of leucylglycine, although this reaction does not distinguish it from the similar leucylpeptidase. A better substrate is alanylglycine, which is split with great rapidity.

According to Grassmann and Schneider (1936; see also Grassmann and Dyckerhoff, 1928) the enzyme requires both a free amino group and a neighboring free carboxyl group. The necessity of imino hydrogen in the peptide linkage was shown by Balls and Köhler (1931). Both amino acids of the peptide must be of the natural configuration (so-called *l*-configuration)* except of course for glycine (which has no optical isomer) and for alanine. Either optical form of alanine is hydrolyzed. Bergmann *et al.* (1935) have explained the foregoing behavior of the enzyme as follows:

The imino hydrogen of the peptide linkage is necessary to permit an enolization, with the resulting formation of a double bond between carbon and nitrogen. This double bond fixes the spatial relations of the two amino acids toward each other. The dipeptide may then be represented as:



Both a *cis* form and a *trans* form of this peptide are possible, but the *cis* form is more probable because of the attraction between amino and carboxyl groups, thus explaining the necessity of both an amino and a carboxyl group, as shown by Grassmann. Furthermore, carbon atoms 1 and 2 in the formula are asymmetric, except in the case of glycine residues. This fixes the position of the two hydrogen atoms, H and H′, in relation to the carboxyl and amino groups, respectively. The optical antipodes of the amino acids in this peptide do not have the hydrogen atoms in the same

* The observed optical rotation of such an amino acid is not necessarily levo. Naturally occurring alanine, for instance, is dextrorotatory.

spatial relations. These two hydrogen atoms must occupy the positions that define the *l*-forms of the amino acids, and this explains the optical specificity of the enzyme. The reason why hydrogen must occupy these particular positions is that, if larger groups were there instead, the enzyme could not (because of steric hindrance) approach the carboxyl and amino groups with which it is supposed to combine. These authors have found that replacement of the hydrogen in question by a methyl group (thus forming an alanyl instead of a glycyl residue) slows down but does not completely stop digestion by dipeptidase. Thus, *d*-alanylglycine (H′ becomes CH_3 in the formula and R′ becomes H) is split by dipeptidase. Also α-aminoisobutyrylglycine is split, though slowly (H′ and R′ in the formula become CH_3). Replacement of the hydrogens by groups larger than methyl completely prevents the enzyme from acting; hence peptides of *d*-leucine are not split.

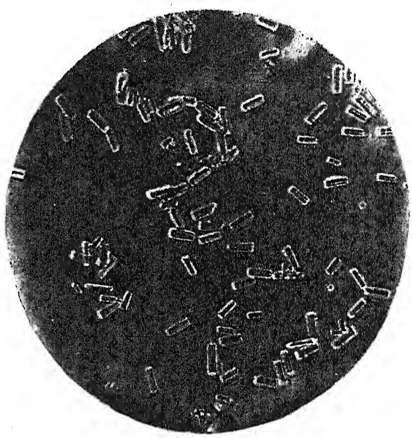


Fig. 8.—Crystals of carboxypeptidase (fourth recrystallization) prepared according to Anson (1937a).

Dipeptidase keeps well in the form of dry preparations such as those of Grassmann, 1929 (see also Grassmann and Klenk, 1929) from yeast and intestinal mucosa dehydrated by acetone and ether. In solution the enzyme is relatively unstable. It is very sensitive to traces of hydrogen sulfide, cyanides, and many heavy metals, and is also inhibited by borate—this precludes the use of borate buffers.

Carboxypeptidase. Carboxypeptidase, discovered by Waldschmidt-Leitz and Purr (1929), occurs in pancreas. The enzyme is evidently formed from an inactive precursor, apparently by the action of trypsin. The pro-enzyme has not been obtained definitely free of possible contamination with trypsinogen so it is impossible to say whether or not enterokinase can also activate it. The addition of enterokinase to preparations made from acetone-dried pancreas by extraction with glycerol does increase the activity of the preparation (Waldschmidt-Leitz and Purr, 1929), but such preparations may contain trypsinogen.

The active peptidase has been crystallized by Anson (1937a) from the exudate of sliced beef pancreas. Typical crystals made by Anson's method are shown in Figure 8. The enzyme protein is a globulin and may be purified by precipitation with a large volume of water.

Carboxypeptidase requires a free carboxyl group on a terminal amino acid of the peptide chain. The carboxyl-bearing amino acid is split off. Not all peptides with free carboxyl groups are attacked, but apparently only those in which the carboxyl group is strongly acid. Peptides containing tyrosine or cysteine at or near the carboxyl end of the chain are most readily split (Waldschmidt-Leitz and Purr, 1929). A free amino group in the substrate is not only unnecessary, but is usually a detriment. Most dipeptides are apparently not acted on at all. Grassmann, Dyckerhoff, and Eibeler (1930) have split glycine from glutathione by means of carboxypeptidase, although glycine in less acid peptides such as leucyldiglycine is not split off. Chloroacetyltyrosine is the usual test substance for this enzyme. Not only is an amino group unnecessary in the substrate, but it is also unnecessary in the enzyme, as appears from the observation of Anson (1937b) that carboxypeptidase is active in solutions containing formaldehyde.

The specific substrate requirements of carboxypeptidase have been examined by Bergmann and Fruton (1937) in a manner similar to that used for dipeptidase. If the substrate (here chloroacetyltyrosine) is written with an enolized peptide bond as on page 262, it is to be expected that most of it exists in the *trans* form, because suitable substrates of carboxypeptidase are peptides in which the basic group is modified by acylation and there is repulsion rather than attraction between it and the carboxyl, or peptides in which the basic group is too far removed by intervening amino acids to attract the carboxyl. The attack of the enzyme is limited to the carboxyl end of the chain. The penultimate amino acid does not appear to be concerned in the reaction, and may therefore be either a natural or an unnatural optical variety, whereas the final amino acid must belong to the naturally occurring series. Thus, the position of H on carbon 2 in the formula is determinant for the action of the enzyme. This was shown by Bergmann, Zervas, and Schleich (1934), who found that both *d*-tyrosylarginine and *l*-tyrosylarginine are split by crude carboxypeptidase. A detailed report of the specificity of crystalline carboxypeptidase was made by Hofmann and Bergmann (1940).

Aminopolypeptidase. This enzyme was discovered in yeast by Grassmann and Dyckerhoff (1928) and was found by Waldschmidt-Leitz and co-workers (1929, 1930) to be a constituent of the "erepsin" of Kestner (Cohnheim). So many contradictory findings on the specificity of aminopolypeptidase have been reported that it is doubtful if the preparations examined have been of comparable purity in many cases. In spite of the scission of dipeptides reported occasionally, it seems likely that the enzyme is a true polypeptidase. Tri- and tetrapeptides are hydrolyzed most readily; longer peptide chains are attacked, although slowly. Preparations of the

intestinal enzyme also digest prolyl peptides, according to Johnson (1937). This may be due to the presence of prolylpeptidase as an impurity or it may be a characteristic of the polypeptidase. In the latter case, it must be assumed that a free amino group is not essential in the substrate, but that a basic imino group may serve as well. When the amino group is acylated, thus losing its basic character, no action by aminopolypeptidase occurs on any known substrate. When a free amino group is present as in leucyldiglycine and similar peptides, the amino acid bearing the amino group is split from the chain. This amino acid must also possess the natural configuration.

Considerable basicity of the initial amino group appears to be required for the action of aminopolypeptidase. It may be that dipeptides are not split because the free carboxyl group is so near the initial amino group that it decreases the basicity of the latter. This reasoning (Balls and Waldschmidt-Leitz, 1929) agrees with the findings of Grassmann (1929) and of Waldschmidt-Leitz and Klein (1928) that certain dipeptide esters and amides, in which the basic properties of the amino group are intensified by removal of the carboxyl group, are split by aminopolypeptidase. Sarcosine inhibits the action of the enzyme, indicating, according to Balls and Köhler (1931, 1932, 1933), the necessity for imino hydrogen at the vulnerable peptide bond.

Peptidases Found to Be Activated by Sulfhydryl or by Metals

Leucylpeptidase. The existence of enzymes specifically adapted to splitting a leucine residue from the amino end of a peptide chain was deduced by Linderstrøm-Lang (1929, 1930) from a comparison of the rates of digestion of di- and tripeptides of alanine and leucine with hog erepsin and extracts of green malt. Johnson, Johnson, and Peterson (1936) showed later that the specific leucine-splitting enzyme was activated by magnesium ions.

Leucine peptidases appear to be widely distributed in nature. Numerous sources such as human and rat intestine, rat carcinoma, several bacteria, and higher plant tissues, have been found by Berger and Johnson (1939a, 1940). Leucine peptidases have been demonstrated by Maschmann (1940a,b) in serum and by Schwimmer (1944) in muscle tissue. Yeasts and molds are less promising sources. The various preparations show about equal rates of hydrolysis for leucylglycine and leucyldiglycine; this indicates no preference by the enzyme between a di- and polypeptide structure. The rate of hydrolysis of alanyl, glycyl, and other peptides by these preparations is very much slower than for leucyl peptides. The optimum hydrogen-ion concentration for leucylpeptidases is quite alkaline (*p*H 8.0–9.5).

The enzymes are activated by both magnesium and manganese ions, the latter frequently leading to faster splitting (Berger and Johnson, 1939).

Mold Polypeptidases. The enzymes of another series isolated by Johnson and Peterson (1935) from molds resemble aminopolypeptidase more closely than do the leucylpeptidases. Dipeptides beginning with leucine or alanine are hydrolyzed slowly, but similar polypeptides of either sort are hydrolyzed rapidly. These enzymes are activated by zinc ions, and in a few cases by nickel.

Catheptic Peptidase. A catheptic peptidase accompanies the tissue proteinase, cathepsin, and according to Anson (1937), is responsible for the more rapid proteolysis observed with crude preparations of cathepsin when hydrogen sulfide or cysteine is present, although cathepsin itself appears not to be influenced by reducing agents. The peptidase has not been isolated. An enzyme corresponding to catheptic peptidase in being activated by hydrogen sulfide was described by Waldschmidt-Leitz, Schäffner, Bek, and Blum (1930) as occurring in spleen. It splits benzoyldiglycine but not benzoylglycine. In a study of the proteolytic activity of spleen, Fruton, Irving, and Bergmann (1941) have demonstrated the multiple nature of this preparation with regard to peptide hydrolysis. At least four components exist, two activated by cysteine, another by ascorbic acid, and a third unaffected by either compound. Two of these enzymes appear to be typical peptidases, while the other two are classified as proteinases. Sulfhydryl peptidases have not yet been found as frequently in nature as the widespread occurrence of sulfhydryl proteinases might suggest. This is possibly because the plant peptidases have had less attention so far.

Peptidases of Grain. Peptidase activity in grain increases greatly during the sprouting period. A dipeptidase from green malt, splitting alanine—and also leucine—dipeptides as well as a leucylpeptidase from the same source have been already discussed. During the growth of the sprout, the dipeptidase is greatly concentrated just short of the tip of the leaf (Linderstrøm-Lang and Holter, 1932).

A peptidase that develops in wheat during sprouting has been investigated by Mounfield (1936, 1938) using filtered aqueous extracts of the whole grain. The peptolytic properties of these preparations are increased by hydrogen sulfide or cyanide, and hence appear to contain an enzyme similar to catheptic peptidase.

Bacterial Peptidases. A group of peptidases, not well separated, has been concentrated by Maschmann (1939, 1939a,b, 1940) from the media of old cultures of several bacteria. The enzyme proteins, precipitated by alcohol or salts, have proved difficult to purify further. The culture filtrates themselves possess very little activity, and require for its development the addition of iron or manganese. The hydrogen-ion optimum

of these peptidases is about pH 7.8. Both dipeptidase and aminopolypeptidase are present.

Maschmann has developed an interesting theory of the constitution of such bacterial peptidases, taking into account the observation that they are inhibited by cyanide and activated by iron. The activation is greater when the addition of iron is preceded by that of cysteine. The addition of iron and cysteine in any other manner has less effect. The inhibition by cyanide indicates that the peptidases are heavy metal complexes (as has been suggested many times about the intestinal peptidases, for the same reason). Unless the culture medium contains sufficient heavy metal, the peptidase proteins will not exist as active enzymes, but in an inactive form as "apopeptidases." The effect of cysteine is explained as dispersing the protein in such a way that the heavy metal may readily enter the molecule at the required site. This concept is supported by the fact that, once activated by iron and later inhibited by cyanide, peptidase activity may be restored in the presence of cyanide by the addition of manganese, whose ions are not removed by cyanide as completely as those of iron. This conception of the composition of peptidases as metal–protein complexes, in which various metals may participate, is a working hypothesis of unusual promise.

BIBLIOGRAPHY

AMBROS, O., and HARTENECK, A. (1929). Über die Proteasen höherer Pflanzen. *Z. physiol. Chem.* 184:93–107.

ANNETTS, M. (1936). The digestion products formed by the action of papain on egg albumin. *Biochem. J.* 30:1807–1814.

ANSON, M. L. (1937). The estimation of cathepsin with hemoglobin and the partial purification of cathepsin. *J. Gen. Physiol.* 20:565–574.

—— (1937a). Carboxypeptidase. I. The preparation of crystalline carboxypeptidase. *J. Gen. Physiol.* 20:663–669.

—— (1937b). Carboxypeptidase. III. The estimation of carboxypeptidase and procarboxypeptidase. *J. Gen. Physiol.* 20:781–786.

—— (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* 22:79–89.

—— (1940). The purification of cathepsin. *J. Gen. Physiol.* 23:695–704.

—— (1941). The sulfhydryl groups of egg albumin. *J. Gen. Physiol.* 24:399–421.

ASENJO, C. F., and CAPELLA DE FERNANDEZ, M. DEL C. (1942). A new protease from *Bromelia pinguin* L. *Science* 95:48–49.

ASTBURY, W. T., DICKINSON, S., and BAILEY, K. (1935). The x-ray interpretation of denaturation and the structure of the seed globulins. *Biochem. J.* 29: 2351–2360.

BALLS, A. K. (1938). Enzyme action in food products at low temperatures. *Brit. Assoc. Refrigeration Proc.*, pp. 10–15.

—— and HALE, W. S. (1938). The preparation and properties of wheat proteinase. *Cereal Chem.* 15:622–628.

—— and HOOVER, S. R. (1937). The milk-clotting action of papain. *J. Biol. Chem.* 121:737–745.

—— and KÖHLER, F. (1931). Über die Wirkungsweise der Peptidasen. *Ber.* 64: 294–301.

BALLS, A. K., and KÖHLER, F., (1932). Über Aminopolypeptidase. *Z. physiol. Chem.* 205:157–170.

—— and —— (1933). Über Aminopolypeptidase. *Z. physiol. Chem.* 219:128–137.

—— and LINEWEAVER, H. (1939). Isolation and properties of crystalline papain. *J. Biol. Chem.* 130:669–686.

—— and —— (1939a). Nature of a group in papain essential to its activity. *Nature* 144:513.

——, SWENSON, T. L., and STUART, L. S. (1935). Assay of papain. *J. Assoc. Official Agr. Chem.* 18:140–146.

——, THOMPSON, R. R., and KIES, M. W. (1941). Bromelin. Properties and commercial production. *Ind. Eng. Chem.* 33:950–953.

—— and WALDSCHMIDT-LEITZ, E. (1929). Concerning animal dipeptidase and polypeptidase. *Am. J. Physiol.* 90:272–273.

BASU, K. P., and NATH, M. C. (1936). Proteinase in the milky juice of *Calotropis gigantea.* Its purification and activation by ascorbic acid and glutathione. *J. Indian Chem. Soc.* 13:34–39.

BERGER, J., and JOHNSON, M. J. (1939). Metal activation of peptidases. *J. Biol. Chem.* 130:641–654.

—— and —— (1939a). The leucylpeptidases of malt, cabbage, and spinach. *J. Biol. Chem.* 130:655–667.

—— and —— (1940). The occurrence of leucylpeptidase. *J. Biol. Chem.* 133:157–172.

BERGMANN, M. (1942). A classification of proteolytic enzymes. In F. F. Nord and C. H. Werkman, *Advances in Enzymology,* Vol. II, pp. 49–68. Interscience, New York.

—— and FRUTON, J. S. (1937). On proteolytic enzymes. XII. Regarding the specificity of aminopeptidase and carboxypeptidase. A new type of enzyme in the intestinal tract. *J. Biol. Chem.* 117:189–202.

—— and —— (1937a). On proteolytic enzymes. XIII. Synthetic substrates for chymotrypsin. *J. Biol. Chem.* 118:405–415.

—— and —— (1938). Some synthetic and hydrolytic experiments with chymotrypsin. *J. Biol. Chem.* 124:321–329.

——, ——, and POLLOK, H. (1939). The specificity of trypsin. *J. Biol. Chem.* 127:643–648.

—— and NIEMANN, C. (1938). On the structure of silk fibroin. *J. Biol. Chem.* 122:577–596.

——, ZERVAS, L., and SCHLEICH, H. (1934). Über proteolytische Enzyme. IV. Mitteilung: Spezifität und Wirkungsweise der sogenannten Carboxy-polypeptidase. *Z. physiol. Chem.* 224:45–51.

——, ——, FRUTON, J. S., SCHNEIDER, F., and SCHLEICH, H. (1935). On proteolytic enzymes. V. On the specificity of dipeptidase. *J. Biol. Chem.* 109:325–346.

BERRIDGE, N. J. (1943). Pure crystalline rennin. *Nature* 151:473–474.

—— (1945). The purification and crystallization of rennin. *Biochem. J.* 39:179–186.

BERSIN, T. (1935). Thiolverbindungen und Enzyme. *Ergeb. Enzymforsch.* 4:68–101.

—— and LOGEMANN, W. (1933). Über den Einfluss von Oxydations- und Reduktionsmitteln auf die Aktivität von Papain. *Z. physiol. Chem.* 220:209–216.

BODANSKY, A. (1924). A study of a milk-coagulating enzyme of *Solanum elaeagnifolium.* *J. Biol. Chem.* 61:365–375.

CALVERY, H. O. (1933). Crystalline egg albumin. The hydrolysis of crystalline egg albumin by pepsin, papain–hydrocyanic acid, and pancreatic proteinase and the subsequent action of some other enzymes on the hydrolysis products produced by these enzymes. *J. Biol. Chem.* 102:73–89.

CARPENTER, D. C., and LOVELACE, F. E. (1943). The isoelectric point of asclepain. *J. Am. Chem. Soc.* 65:2364–2365.

CASTAÑEDA, M., GAVARRON, F. F., and BALCAZAR, M. R. (1942). On a new protease from *Pileus mexicanus.* *Science* 96:365–366.

CHARGAFF, E., and BENDICH, A. (1943). On the coagulation of fibrinogen. *J. Biol. Chem.* 149:93–110.

DYCKERHOFF, H. (1940). Über die Gerinnung des Blutes. In F. F. Nord and R. Weidenhagen, *Handbuch der Enzymologie*, Vol. I, pp. 633–648. Akadem. Verlagsgesellschaft, Leipzig.

EAGLE, H. (1937). The coagulation of blood by snake venoms and its physiologic significance. *J. Exptl. Med.* 65:613–639.

—— and HARRIS, T. N. (1937). Studies in blood coagulation. V. The coagulation of blood by proteolytic enzymes (trypsin, papain). *J. Gen. Physiol.* 20: 543–560.

EDLBACHER, S., GOLDSCHMIDT, E., and SCHLÄPPI, V. (1934). Über die Enzyme des Gehirns. *Z. physiol. Chem.* 227:118–123.

FRUTON, J. S., and BERGMANN, M. (1939). The specificity of pepsin. *J. Biol. Chem.* 127:627–641.

—— and —— (1940). The activation of papain. *J. Biol. Chem.* 133:153–156.

——, IRVING, G. W., JR., and BERGMANN, M. (1941). On the proteolytic enzymes of animal tissues. III. The proteolytic enzymes of beef spleen, beef kidney, and swine kidney. Classification of the cathepsins. *J. Biol. Chem.* 141:763–774.

GODDARD, D. R., and MICHAELIS, L. (1934). A study on keratin. *J. Biol. Chem.* 106:605–614.

GOTTSCHALL, G. Y. (1944). The activation of papain during the digestion of meat. *Food Research* 9:6–10.

GRASSMANN, W. (1929). Untersuchungen über die Spezifität proteolytischer Pflanzenenzyme. Habilitationsschrift, Munich.

—— and DYCKERHOFF, H. (1928). Über die Spezifität der Hefe-Peptidasen. *Ber.* 61:656–670.

—— and —— (1928a). Über die Proteinase und die Polypeptidase der Hefe. *Z. physiol. Chem.* 179:41–78.

——, ——, and EIBELER, H. (1930). Über die enzymatische Spaltung des Glutathions. I. *Z. physiol. Chem.* 189:112–120.

——, ——, and SCHOENEBECK, O. v. (1929). Über die enzymatische Spaltbarkeit der Prolin-Peptide. *Ber.* 62:1307–1310.

—— and KLENK, L. (1929). Zur Frage der Einheitlichkeit tierischer und pflanzlicher Dipeptidase. *Z. physiol. Chem.* 186:26–49.

—— and SCHNEIDER, F. (1936). Proteasen. *Ergeb. Enzymforsch.* 5:79–116.

GREENBERG, D. M., and WINNICK, T. (1940). Plant proteases. I. Activation-inhibition reactions. II. pH-activity curves. III. Kinetic properties. *J. Biol. Chem.* 135:761–787.

GREENSTEIN, J. P. (1937). Studies of multivalent amino acids and peptides. IX. The synthesis of *l*-cystinyl-*l*-cystine. *J. Biol. Chem.* 121:9–17.

HANKINSON, C. L. (1943). The preparation of crystalline rennin. *J. Dairy Sci.* 26:53–62.

HEPBURN, J. S., and JONES, F. M. (1927). Enzymes of the pitcher liquor of the *Sarraceniaceae*. *Trans. Wagner Free Inst. Sci. Phila.* 11:49–68.

HERRIOTT, R. M. (1935). Acetylation of tyrosine in pepsin. *J. Gen. Physiol.* 19: 283–299.

—— (1937). Inactivation of pepsin by iodine and the isolation of diiodo-tyrosine from iodinated pepsin. *J. Gen. Physiol.* 20:335–352.

—— (1938). Isolation, crystallization, and properties of swine pepsinogen. *J. Gen. Physiol.* 21:501–540.

—— (1938a). Kinetics of the formation of pepsin from swine pepsinogen and identification of an intermediate compound. *J. Gen. Physiol.* 22:65–78.

—— (1941). Isolation, crystallization, and properties of pepsin inhibitor. *J. Gen. Physiol.* 24:325–338.

HERRIOTT, R. M., and NORTHROP, J. H. (1934). Crystalline acetyl derivatives of pepsin. *J. Gen. Physiol.* **18**:35–67.

HOFMANN, K., and BERGMANN, M. (1939). The specificity of trypsin. II. *J. Biol. Chem.* **130**:81–86.

—— and —— (1940). The specificity of carboxypeptidase. *J. Biol. Chem.* **134**:225–235.

HOLTER, H., and LINDERSTRØM-LANG, K. (1933). Beiträge zur enzymatischen Histochemie. III. Über die Proteinasen von *Drosera rotundifolia*. *Z. physiol. Chem.* **214**:223–240.

HORWITT, M. K. (1944). Observations on the first stages of casein hydrolysis by chymotrypsin and trypsin. *J. Biol. Chem.* **156**:421–425.

IRVING, G. W., JR., FRUTON, J. S., and BERGMANN, M. (1941). Kinetics of proteinase action. Application to specificity problems. *J. Biol. Chem.* **138**: 231–242.

JAFFÉ, W. G. (1943). Hurain, a new plant protease from *Hura crepitans*. *J. Biol. Chem.* **149**:1–7.

JANSEN, E. F., and BALLS, A. K. (1941). Chymopapain: A new crystalline proteinase from papaya latex. *J. Biol. Chem.* **137**:459–460.

JOHNSON, M. J. (1937). Specificity of intestinal aminopolypeptidase. *J. Biol. Chem.* **122**:89–97.

—— (1941). Isolation and properties of a pure yeast polypeptidase. *J. Biol. Chem.* **137**:575–586.

——, JOHNSON, G. H., and PETERSON, W. H. (1936). The magnesium-activated leucyl peptidase of animal erepsin. *J. Biol. Chem.* **116**:515–526.

—— and PETERSON, W. H. (1935). The peptidase system of *Aspergillus parasiticus*. *J. Biol. Chem.* **112**:25–34.

KLEINER, I. S., and TAUBER, H. (1932). Studies on rennin. II. The isolation of prorennin. *J. Biol. Chem.* **96**:755–758.

KREBS, H. A. (1930). Über "Aktivierung" proteolytischer Fermente. *Naturwissenschaften* **18**:736.

KUNITZ, M. (1938). Formation of trypsin from trypsinogen by an enzyme produced by a mold of the genus *Penicillium*. *J. Gen. Physiol.* **21**:601–620.

—— (1938a). Formation of new crystalline enzymes from chymotrypsin. Isolation of beta and gamma chymotrypsin. *J. Gen. Physiol.* **22**:207–237.

—— (1939). Formation of trypsin from crystalline trypsinogen by means of enterokinase. *J. Gen. Physiol.* **22**:429–446.

—— and NORTHROP, J. H. (1934). Autocatalytic activation of trypsinogen in the presence of concentrated ammonium or magnesium sulfate. *Science* **80**:190.

—— and —— (1935). Crystalline chymo-trypsin and chymo-trypsinogen. I. Isolation, crystallization, and general properties of a new proteolytic enzyme and its precursor. *J. Gen. Physiol.* **18**:433–458.

—— and —— (1936). Isolation from beef pancreas of crystalline trypsinogen, trypsin, a trypsin inhibitor, and an inhibitor-trypsin compound. *J. Gen. Physiol.* **19**: 991–1007.

LANDIS, Q., and FREY, C. N. (1938). A sensitive method for the determination of proteolytic activity. *Cereal Chem.* **15**:91–101.

LINDERSTRØM-LANG, K. (1929). Über Darmerepsin. *Z. physiol. Chem.* **182**: 151–174.

—— (1930). Über die Einheitlichkeit der Darmdipeptidase. *Z. physiol. Chem.* **188**: 48–68.

—— and DUSPIVA, F. (1935). Beiträge zur enzymatischen Histochemie. XVI. Die Verdauung von Keratin durch die Larven der Kleidermotte (*Tineola biselliella* Humm.). *Z. physiol. Chem.* **237**:131–158.

—— and HOLTER, H. (1932). Beiträge zur enzymatischen Histochemie. II Über die Peptidaseverteilung in Wurzel und Blattkeim des Malzkornes. *Z. physiol. Chem.* **204**:15–53.

LINDERSTRØM-LANG, K., and SATO, M. (1929). Die Spaltung von Glycylglycin, Alanylglycin und Leucylglycin durch Darm- und Malzpeptidasen. *Z. physiol. Chem.* **184**:83–92.

LINEWEAVER, H., and HOOVER, S. R. (1941). A comparison of the action of crystalline papain on native and urea-denatured proteins. *J. Biol. Chem.* **137**: 325–335.

—— and SCHWIMMER, S. (1941). Some properties of crystalline papain: Stability toward heat, pH and urea; pH optimum with casein as substrate. *Enzymologia* 10:81–86.

MASCHMANN, E. (1939). Zur Kenntnis der Proteasen des *B. sporogenes.* Über eine neue Proteinase und über aktivierbare Peptidasen. *Biochem. Z.* **300**: 89–112.

—— (1939a). Über Bakterienproteasen. XII. Zur Kenntnis der Dipeptidasen anaerober Bakterien. *Biochem. Z.* **302**:332–368.

—— (1939b). Über Bakterienproteasen. XIII. Über die Abwesenheit einer für die Wirksamkeit notwendigen Carbonylgruppe in den Anaerodipeptidasen. *Biochem. Z.* **303**:145–151.

—— (1940). Über Bakterienproteasen. XVI. Zur Kenntnis der Aminopolypeptidasen anaerober Bakterien. *Biochem. Z.* **307**:1–34.

—— (1940a). Über die Aktivierung der Serumpeptidasen. *Naturwissenschaften* **28**: 765.

—— (1940b). Zur Aktivierung der Serumpeptidasen. *Naturwissenschaften* **28**:780–781.

MENDEL, L. B., and BLOOD, A. F. (1910). Some peculiarities of the proteolytic activity of papain. *J. Biol. Chem.* **8**:177–213.

MILLER, L. (1939). Digestion of crystalline lactoglobulin by crystalline trypsin and chymotrypsin. *Compt. rend. trav. lab. Carlsberg, Ser. chim.* **23**:45–53.

MOUNFIELD, J. D. (1936). The proteolytic enzymes of sprouted wheat. *Biochem. J.* **30**:549–557.

—— (1936a). The proteolytic enzymes of sprouted wheat. II. *Biochem. J.* **30**:1778–1786.

—— (1938). The proteolytic enzymes of sprouted wheat. III. *Biochem. J.* **32**:1675–1684.

NORRIS, E. R., and ELAM, D. W. (1940). Preparation and properties of crystalline salmon pepsin. *J. Biol. Chem.* **134**:443–454.

NORTHROP, J. H. (1930). Crystalline pepsin. I. Isolation and tests of purity. II. General properties and experimental methods. *J. Gen. Physiol.* **13**:739–780.

—— (1931). The presence of a gelatin-liquefying enzyme in crude pepsin preparations. *J. Gen. Physiol.* **15**:29–43.

—— (1932). Pepsin activity units and methods for determining peptic activity. *J. Gen. Physiol.* **16**:41–58.

—— (1939). *Crystalline Enzymes.* Columbia Univ. Press, New York.

—— and KUNITZ, M. (1930). Solubility curves of mixtures and solid solutions. *J. Gen. Physiol.* **13**:781–791.

—— and —— (1932). Crystalline trypsin. I. Isolation and tests of purity. II. General properties. III. Experimental procedure and methods of measuring activity. *J. Gen. Physiol.* **16**:267–321.

OLCOTT, H. S., SAPIRSTEIN, L. A., and BLISH, M. J. (1943). Stability of wheat gluten dispersions toward reducing agents in the presence and absence of a gluten proteinase. *Cereal Chem.* **20**:87–97.

PETERMANN, M. L. (1942). The action of papain on beef serum pseudoglobulin and on diphtheria antitoxin. *J. Biol. Chem.* **144**:607–616.

PHILPOT, J. ST. L., and SMALL, P. A. (1938). The action of nitrous acid on pepsin. *Biochem. J.* **32**:542–551.

PLENTL, A. A., and PAGE, I. H. (1944). On the enzymatic specificity of renin. I. The proteinase components of renin preparations and their relation to renin activity. *J. Biol. Chem.* **155**:363–378.

PURR, A. (1935). The activation phenomena of papain and cathepsin. *Biochem. J.* 29:13–20.

SCHEPOWALNIKOW, N. P. (1899). Die Physiologie des Darmsaftes. *Jahresber. Fortschr. Tier-Chem.* 29:378–380.

SCHWANN, T. (1836). Über das Wesen des Verdauungsprocesses. *Arch. Anat. Physiol. wiss. Med.*, 90–138.

SCHWIMMER, S. (1944). Comparison of crude and purified preparations of a leucylpeptidase associated with beet muscle. *J. Biol. Chem.* 154:361–366.

SMITH, E. L., and BERGMANN, M. (1944). The peptidases of intestinal mucosa. *J. Biol. Chem.* 153:627–651.

STICKLAND, L. H. (1929). The bacterial decomposition of formic acid. *Biochem. J.* 23:1187–1198.

SUMNER, J. B. (1926). The isolation and crystallization of the enzyme urease. Preliminary paper. *J. Biol. Chem.* 69:435–441.

—— and DOUNCE, A. L. (1937). Crystalline catalase. *J. Biol. Chem.* 121:417–424.

—— and —— (1939). Catalase. II. *J. Biol. Chem.* 127:439–447.

TAUBER, H., and KLEINER, I. S. (1932). Studies on rennin. I. The purification of rennin and its separation from pepsin. *J. Biol. Chem.* 96:745–753.

—— and —— (1933). Die milchkoagulierenden Enzyme der Magenschleimhaut und ihre Zymogene. *Z. physiol. Chem.* 220:205–208.

—— and —— (1934). The chemical nature of rennin. *J. Biol. Chem.* 104:259–270.

TISELIUS, A. (1937). A new apparatus for electrophoretic analysis of colloidal mixtures. *Trans. Faraday Soc.* 33:524–531.

—— and ERIKSSON-QUENSEL, I. (1939). Some observations on peptic digestion of egg albumin. *Biochem. J.* 33:1752–1756.

——, HENSCHEN, G. E., and SVENSSON, H. (1938). Electrophoresis of pepsin. *Biochem. J.* 32:1814–1818.

VAN SLYKE, D. D. (1913). The gasometric determination of aliphatic amino nitrogen in minute quantities. *J. Biol. Chem.* 16:121–124.

VINES, S. H. (1903). Proteolytic enzymes in plants. *Ann. Botany* 17:237–264.

WALDSCHMIDT-LEITZ, E. (1924). Über Enterokinase und die tryptische Wirkung der Pankreasdrüse. *Z. physiol. Chem.* 132:181–237.

—— (1930). Structure of proteins from the basis of enzyme analysis. *Chem. Weekblad* 27:266–271.

—— and BALLS, A. K. (1930). Zur Kenntnis der Amino-Polypeptidase aus Darm-Schleimhaut. *Ber.* 63:1203–1211.

——, ——, and WALDSCHMIDT-GRASER, J. (1929). Über Dipeptidase und Polypeptidase aus Darm-Schleimhaut. *Ber.* 62:956–962.

—— and KLEIN, W. (1928). Über Spezifität und Wirkungsweise von Erepsin, Trypsin und Trypsin-Kinase. *Ber.* 61:640–645.

—— and PURR, A. (1929). Über Proteinase und Carboxy-Polypeptidase aus Pankreas. *Ber.* 62:2217–2226.

——, SCHÄFFNER, A., BEK, J. J., and BLUM, E. (1930). Über das proteolytische System in tierischen Organen. *Z. physiol. Chem.* 188:17–47.

WALTI, A. (1938). Crystalline ficin. *J. Am. Chem. Soc.* 60:493.

WARBURG, O., and CHRISTIAN, W. (1943). Isolierung und Kristallisation des Gärungsferments Zymohexase. *Biochem. Z.* 314:149–176.

WILLSTÄTTER, R., and BAMANN, E. (1929). Über die Proteasen der Magenschleimhaut. Erste Abhandlung über die Enzyme der Leukocyten. *Z. physiol. Chem.* 180:127–143.

——, GRASSMANN, W., and AMBROS, O. (1926). Blausäure-Aktivierung und -Hemmung pflanzlicher Proteasen. *Z. physiol. Chem.* 151:286–306.

—— and WALDSCHMIDT-LEITZ, E. (1921). Alkalimetrische Bestimmung von Aminosäuren und Peptiden. *Ber.* 54:2988–2993.

—— and —— (1923). Über Pankreaslipase. Zweite Abhandlung über Pankreasenzyme. *Z. physiol. Chem.* 125:132–198.

WINNICK, T. (**1944**). General characteristics of the partial hydrolysis products from the action of proteolytic enzymes on casein. *J. Biol. Chem.* **152**:465–473.

——, CONE, W. H., and GREENBERG, D. M. (**1944**). Experiments on the activation of ficin. *J. Biol. Chem.* **153**:465–470.

——, DAVIS, A. R., and GREENBERG, D. M. (**1940**). Physicochemical properties of the proteolytic enzyme from the latex of the milkweed, *Asclepias speciosa* Torr. Some comparisons with other proteases. *J. Gen. Physiol.* **23**:275–308.

CHAPTER IX

ROLE OF PROTEASES IN BAKING

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In order to evaluate the practical significance of proteolytic enzymes in breadmaking and related processes, the available evidence must be examined to determine: whether proteases are present in wheat, in flour, in malt products, and in yeast; if present, in what manner the action of these enzymes may affect baking behavior; and whether such proteolytic action as may occur is of sufficient magnitude to be of practical importance. It will be convenient to take up these points as they apply to sound wheat and flour produced therefrom, to malt products or to sprout-damaged grain, to yeast, and to wheat damage by insects.

SOUND WHEAT AND FLOUR

It has long been known that wheat and flour contain enzymes capable of hydrolyzing proteins or their split-products. Thus, Balland (1884) reported the presence of an enzyme system in wheat germ which was capable of liquefying gluten. Enzyme action was evidenced by the fact that liquefaction was not observed when the germ was boiled in water prior to addition to the gluten. Ford and Guthrie (1908) carried out a study of the proteases of wheat flour, using a variety of techniques. It was demonstrated that, when flour was mixed with a gelatin solution, liquefaction of the gelatin took place on standing, even when a disinfectant was used to eliminate possible bacterial or mold action. The same results were obtained when flour extracts were used. These authors also observed that, when an active protease was added to bread doughs, they became progressively more fluid. A fair correlation was obtained between the apparent proteolytic activity, as judged by gelatin liquefaction, and the tendency of doughs to soften during fermentation.

Baker and Hulton (1908) also obtained qualitative evidence of the presence of proteases in flour and observed that the apparent activity multiplied several times during the germination of wheat.

Other techniques have commonly been used in the investigation of flour proteases. Thus, Swanson and Tague (1916) employed the Sørensen formol-titration method to determine the rate of increase in amino nitrogen in autolyzing flour extracts. Despite the fact that autolysis was continued for long periods of time, a relatively small increase in amino nitrogen was observed. It should be pointed out, however, that the absence of an appreciable increase in the end-products of hydrolysis does not necessarily imply that there may not have been a significant alteration in the structure of the whole protein. Thus, for example, the hydrolysis of one peptide linkage in the center of a protein molecule would reduce the size of the molecule by a factor of two, whereas the corresponding increase in free amino nitrogen might well be so slight as to be unmeasurable.

Stockham (1920) studied the action of flour extracts on a gelatin substrate and observed that the concentration of proteases, as judged by this technique, increased with decrease in flour grade and that the concentration was highest in the germ end of the kernel. Cairns and Bailey (1928) used a number of chemical procedures to determine the course of proteolysis in flour suspensions. Their results indicated that precipitation of pro-

TABLE I

INCREASE IN AMINO NITROGEN DURING DIGESTION OF DIFFERENT FLOUR GRADES, MILLED FROM HARD RED SPRING WHEAT, AS DETERMINED BY SØRENSEN'S AND FOREMAN'S TITRATION METHODS (From Cairns and Bailey, 1928)

Flour stream	Increase in "amino" N per 10 g flour during digestion for 48 hrs at 37°C	
	Sørensen's method	Foreman's method
	mg	*mg*
Middlings		
First	0.4	—
Second	0.5	1.0
Third	0.5	—
Fourth	0.5	1.0
Fifth	0.8	—
Sixth	0.7	—
Break		
First	0.7	—
Second	1.1	1.4
Third	0.9	—
Fourth	1.8	2.2
Fifth	2.8	4.8
Tailings		
First	0.9	1.7
Second	1.8	3.9
Sizing	0.5	1.7
Red-dog	3.7	11.0
Bran and shorts duster	2.1	4.3

tein by cupric hydroxide or by tungstic acid was of value in following protein disintegration. In general, of the methods that measure increase in free amino nitrogen or free carboxyl, the Sørensen procedure was the best suited to measurements of flour proteolysis. They confirmed the findings of Stockham (1920) that proteolysis increases with decrease in flour grade. Typical of their data are the values given in Table I. Further evidence of the presence of proteases in patent flours was obtained by extracting such flours with water, adding safranine to the extract, and collecting the precipitate formed—a technique proposed by Marston (1923). The precipitates obtained by Cairns and Bailey showed marked proteolytic activity.

A valuable contribution to our knowledge of wheat and flour proteinases was made independently by Jørgensen (1935) and by Balls and Hale (1935). In both the Copenhagen and Washington laboratories it was proved beyond question that proteinase occurred in these products and that the enzyme was of the papain type; it was characterized by its activation by reducing agents such as cysteine, and inhibition by bromate, persulfate, iodoacetic acid, and other oxidants. Balls and Hale (1936, 1938) concentrated this enzyme from bran and whole wheat, and Hale (1939) prepared it from patent flour. The characteristic behavior of these proteinase concentrates is shown in Table II.

TABLE II

ACTIVATION AND INHIBITION OF THE PROTEINASE FROM FLOUR (From Hale, 1939)

Volume of enzyme solution, ml	No additions	Activity[1] after treatment for 30 minutes				
		Cysteine	$KBrO_3$		$K_2S_2O_8$	
		10 mg	1 mg	2 mg	1 mg	2 mg
1	0.8	1.1	—	—	—	—
2	2.0	2.3	0.5	0.2	0.3	0.3

Volume of enzyme solution, ml	$NaVO_3$		Iodoacetic acid[2]	Ascorbic acid	Cystine	
	1 mg	2 mg	$M/100$	1 mg	2 mg	10 mg
2	0.8	0.3	0.2	0.4	0.7	1.3

[1] Activity values refer to mg protein N in the amount of reference preparation showing the same activity.

[2] Concentration in the enzyme solution.

It may be safely concluded that proteases are present in sound wheat and flour, although much of the evidence indicates that the concentration of these enzymes is low.

The chief interest in proteases, as far as milling and baking technology is concerned, lies in the question of whether or not the concentration of such enzymes normally present in flour is sufficiently great to effect a sig-

nificant hydrolysis of gluten proteins under normal baking conditions. As noted above, flour proteinases are of the papain type (Hale, 1939). It has been repeatedly and convincingly demonstrated that the addition of papain to a dough, or to gluten washed therefrom, brings about a softening and stickiness in the dough—the effect becoming more pronounced with extended time of action—and a disaggregation of the gluten proteins (see, for example, Flohil, 1936; Elion, 1943). It seems reasonable to expect that flour proteinases, if present in sufficient concentration and under conditions optimal for their activity, would have a similar effect.

In recent years the question of whether or not proteinases are responsible for the slackening noted with certain flours during fermentation has been vigorously argued. Impetus for many studies on this point came from the work of Jørgensen (1935, 1935a, 1936, 1939) and of Balls and Hale (1935, 1936, 1936a, 1938). In 1935, Jørgensen advanced the hypothesis that oxidizing agents that have an "improving" action in bread baking have this effect because they inhibit proteinase action; otherwise such action would bring about excessive gluten hydrolysis with concomitant lowering of gas-retaining capacity in the fermenting doughs. He showed experimentally that flour proteinases are inactivated by bromate and iodate, whereas chlorate is without effect. The activity of the proteinases of malted wheat and of wheat germ was also reduced by the addition of bromate. Bromate and iodate are flour improvers; chlorate does not have such improving action. Therefore, reasoning by analogy, Jørgensen propounded the hypothesis of the mechanism of bromate action cited above.

Independently, and about the same time, Balls and Hale (1935) came to the same conclusion. In a later publication (1936a), these authors stated:

> Experiments at the U. S. Bureau of Chemistry and Soils have shown that the autolysis of flour proteins is increased by the addition of cysteine or glutathione, thus indicating that the protein-splitting enzyme is of the papain type (Balls and Hale, 1935). The enzyme is largely resident in the bran and germ. Similar activation of the hydrolysis of casein by the water-soluble enzyme confirmed this conclusion (Balls and Hale, 1936). The activity of oxidizing agents in improving the appearance of bread, the effect of oxidants used in bleaching flour, and the improvement that flour undergoes on storage in air can be adequately explained as resulting from the oxidation of the cysteine-like activator. A diminution in the proteolytic activity of the dough is a consequence of such treatment, with the result that the dough of the treated flour does not become as soft as the dough of the untreated and proteolytically more active flour. The difficulties found in preparing a light, spongy loaf of bread from whole wheat flour, or from flour containing wheat-germ are probably in part due to the fact that such materials are much richer in proteinase (or else in activator) than is normal white flour.

As a corollary to the observation that oxidative improvers decrease proteolytic activity in doughs, it was also suggested by Jørgensen and by

Balls and Hale that the harmful effects of reducing agents could be accounted for by their action as proteinase activators. Thus Jørgensen, in 1936, stated:

> . . . *wheat flour is a material which contains powerful proteinases. Usually, however, these powerful proteinases do not manifest their existence because they lack suitable activators. In the presence of a suitable activator, glutathione, for instance, the great power of the proteinases of the wheat flour manifests itself very clearly.*
>
> Realizing that flour-proteinases when activated are very powerful and *that an activation certainly takes place during the fermentation time (because yeast is an important source of substances which are able to activate flour proteinases)*, one can no longer wonder that substances such as $KBrO_3$, which are able to depress the proteinase-activity, exhibit a strong influence on baking strength.

This argument was set forth as an answer to the question of how the apparently limited concentration of proteinases present in patent flour milled from normal wheats could have a significant effect on dough properties. It was demonstrated that the addition of glutathione or of boiled yeast water brought about an apparent dough liquefaction and resulted in the production of a loaf of small volume with very inferior internal characteristics. Note, however, that there is no direct evidence here that the action of reducing agents takes place through their indirect effect as proteinase activators. It was only shown that, as glutathione or yeast extracts—both devoid of proteolytic activity—were added to fermented doughs, these unfavorable characteristics were obtained.

In the same year, Balls and Hale (1936a) also reported the results of a study of the effect of reducing agents on flour proteins. As a result of experiments involving the addition of reducing agents to gluten, it was concluded: "We are apparently dealing with a reduction of the gluten proteins, for the amount of reductant required is far greater than that needed to activate a small quantity of proteinase." The dispersion of gluten observed on treatment with reducing agents was believed not to be an effect of activated proteinase because the reaction appeared to take place rapidly, and because the end-products, in this case dispersed gluten, showed no further change on continued standing. These authors came to the conclusion that it is probable that reducing agents act in two distinct ways: the addition of small amounts of reducing agents activates the proteinase present in doughs, while further additions will bring about direct chemical action on the gluten as well.

Two years later, Balls and Hale (1938) reported the results of a study in which the proteinase of wheat was concentrated and its properties further investigated. Additional and conclusive evidence was obtained that wheat proteinases are of the papain type. The partially purified enzyme preparation became inactive on standing in air but could be reactivated by the addition of cysteine. The active enzyme digested casein,

clotted milk, and lowered the viscosity of gelatin dispersions. It was also inactivated by several oxidative bread improvers—namely, bromate, persulfate, and metavanadate. Again, like papain, the wheat proteinase was inactivated by iodoacetic acid. In this report these authors returned to the conclusion: "Since it may be fairly concluded that the proteinase of wheat is an enzyme similar to papain, its inactivation by oxidizing agents such as the usual bread improvers still seems to explain adequately the effect of the latter in the dough."

Despite the analogy between the action of oxidizing agents on wheat proteinase and their chemical effect as bread improvers, there still remain some discrepancies which have not been satisfactorily reconciled with this suggested mechanism of improver action. The possibility that proteolytic activity might be the factor in wheat germ responsible for its deleterious effect on dough characteristics and on bread quality was considered by Geddes (1930). However, he rejected the hypothesis, reasoning that the effect of proteolysis should be cumulative with time, and that such was not the case. Quite to the contrary, progressively less effect was noted from germ addition as fermentation was prolonged. This behavior could not be reconciled with the assumption that the harmful effects of germ are due either to its high proteinase content or to a high concentration of proteinase activators.

Ford and Maiden (1938) raised a similar objection, based on farinograph studies of doughs to which glutathione or papain had been added. The modes of action of the two preparations were distinctly different. The glutathione caused an immediate softening effect which did not increase with time, whereas, when papain was added, the doughs softened immediately, and, in addition, showed a pronounced progressive softening with the passing of time. If glutathione acted primarily as an activator of flour proteinases, it would be expected that enzymatic action would bring about a continuous alteration of dough properties. Since this was evidently not the case, Ford and Maiden concluded that the major effect of glutathione was exerted directly upon the flour proteins.

Baker and Mize (1937, 1939) have reported results of investigations on the effects of mixing doughs in vacuum and in the presence of air, oxygen, nitrogen, and hydrogen—with and without the addition of chemical oxidants. Whenever oxygen or an excess of an inorganic oxidizing agent was added, the dough became short and sticky, and the quality of the bread produced from that dough was impaired. The deleterious effect of excess bromate was found to be promoted by mixing. In the absence of action brought about either by mechanical mixing or by the mixing resulting from yeast fermentation, bromate had little effect.

Freilich and Frey (1939, 1943) demonstrated conclusively that the

addition of bromate produced effects over and beyond those that could be attributed to proteinase inactivation. As increasing amounts of bromate were added, loaf volume progressively decreased and internal characteristics became poorer. The "excess bromate effect," which could be largely eliminated by remixing the doughs after fermentation, is an important argument against the hypothesis that oxidizing improvers exert their effect solely through inhibition of flour proteinases. If the hypothesis were true, it would follow that any amount of improver great enough to bring about complete inactivation would produce bread of optimal quality. In other words, loaf volume or loaf characteristics should be progressively improved as the amount of bromate is increased until complete inactivation of the proteinases has been effected, when more bromate should produce no further improvement. This is not the case. For most flours there is an optimum level of bromate treatment, and the quality of bread produced falls off with either smaller or larger amounts of bromate. This was brought out by Shen and Geddes (1942), who showed further that the loaf volume response to bromate was greater with decreasing flour grade; the optimum bromate treatment was progressively higher for clear and low-grade flours than for patent flours. These authors drew no definite conclusion as to the mechanism of the action of oxidizing agents, but expressed the belief that the analytical techniques available at the present time are inadequate for a satisfactory solution of this problem.

Sullivan, Howe, Schmalz, and Astleford (1940) made an exhaustive study of the action of oxidizing and reducing agents on flour, and pointed out that there is a pronounced difference in the rate of action of various oxidizing improvers. The action of iodate is extremely rapid, its presence being noticeable immediately after mixing, whereas with bromate the results are not apparent until some time after fermentation has started. Lesser quantities of iodate are required to give the same effect. Vanadate gives results similar to those of bromate and iodate, but in still smaller amounts than iodate. No evidence could be found that the various oxidants had any influence on amylase activity, lipides, starch, or sugars. Some qualitative results indicating a direct action of oxidizing and reducing agents on gluten proteins were described. For example, washed gluten was rapidly peptized after the addition of a small amount of reducing agent (0.1 ml of thioglycolic acid was added to the wet gluten from 240 g flour). Similar results were obtained with cysteine and glutathione. These authors conclude: "In our opinion, the action of these SH compounds is not on any proteolytic enzyme because one would expect the latter to have been washed away, to some extent at least, in recovering the gluten. . . . It is difficult to prove definitely that the activation of the proteolytic enzymes is a secondary effect until we have a better way of measuring proteolysis."

Olcott, Sapirstein, and Blish (1943) have recently reported findings that conflict with the hypothesis that oxidizing and reducing agents affect dough properties indirectly through their action as proteinase inhibitors and activators. It was found that, when gluten was washed from bakers' patent flour, some or all of the proteinase originally present in the flour was adsorbed on the gluten. When the glutens were dispersed in dilute acetic acid and allowed to stand, their physical and chemical composition underwent a slow change with time as evidenced by progressive decrease in viscosity and increase in nonprotein nitrogen and in formol-titration values. When the glutens had been previously heated to 100°C for 5 to 10 minutes, these properties remained essentially constant.

Of particular interest to the present discussion was the observation that, when gluten dispersions were heat-treated and the proteinase thus inactivated, the addition of reducing agents such as cysteine, thioglycol, sodium bisulfite, and potassium cyanide brought about a prompt and pronounced alteration in physical properties as shown by viscosity changes. On the other hand, formol-titration values remained constant, and the absence of enzymatic activity was thus proved. The decrease in the original formol-titration value (see Table III) in the presence of monothioglycol is caused by the reaction of the thioglycol with formaldehyde, which releases alkali. Typical data are shown in Table III.

TABLE III

COMPARISON OF ENZYMATIC CHANGE IN UNHEATED GLUTEN DISPERSION WITH EFFECT OF MONOTHIOGLYCOL ON HEATED GLUTEN DISPERSION AT 25°C
(From Olcott, Sapirstein, and Blish, 1943)

All solutions contained 6.3 mg N per ml in 0.1 N acetic acid, pH 3.7

Dispersion	Relative viscosity		Formol titration[1]	
	1 hr	42 hrs	0 hr	42 hrs
Gluten	4.0	2.8	1.3	2.0
Heated gluten[2]	4.6	4.6	1.3	1.3
Heated gluten + 0.01 M monothioglycol	3.9	3.1	1.1	1.1

[1] As ml 0.1 N NaOH required per g of gluten.
[2] Heated 5 minutes at 100°C.

As a result of these studies, Olcott, Sapirstein, and Blish concluded that the importance of this proteinase system in baking technology is problematical. The activity observed was low even after activation. In evaluating this point, the reader must remember that there is a strong possibility that not all of the proteinase originally present in flour was adsorbed by the gluten and thus recovered by the techniques used in these studies. The

evidence considered here shows that, in addition to activating proteinases, reducing agents have profound effects on gluten dispersions, even though the proteinase adsorbed on the gluten proteins has been entirely inactivated. Olcott *et al.* concur in the idea advanced by Ford and Maiden (1938; see also Balls and Hale, 1936) that the effect of reducing agents on gluten and dough characteristics is primarily a direct chemical action on the protein molecule and only secondarily a result of proteinase activation.

These discrepancies between the proteinase theory and the observed action of reducing and oxidizing agents in doughs have been recently reviewed by Sandstedt and Fortmann (1943). These authors state:

The characteristic course of the action of oxidation and reduction in doughs seems to us to furnish convincing evidence that the manifestations of reduction and of oxidation in doughs are not due to the activation and inactivation of proteolytic enzymes. It is well known that the oxidation of a dough by bromate is slow and is progressive over a considerable period of time. If doughs made from unoxidized flour are treated with normal quantities of $KBrO_3$ (1 to 2 mg %) the oxidizing action is not noticeable in the dough until after a considerable period of fermentation; there is a gradual development of oxidation or gradual disappearance of reduced character. If the reduced character is the result of proteolytic disintegration of the protein, its disappearance should mean a resynthesis of the gluten protein. As compared with the action of $KBrO_3$, the action of KIO_3 or $NaClO_2$ is exceedingly rapid and is noticeable in the dough as it comes from the mixer (Sullivan, Howe, Schmalz, and Astleford, 1940; Baker and Mize, 1941), although even with these agents there is a pronounced increase in oxidation with time.

These workers made a thorough baking study in which they used a second clear flour which undoubtedly contained a much higher concentration of proteinase than that normally present in patent or straight-grade flours. Oxidizing and reducing agents were added and the effects compared with those obtained by the addition of papain. It was shown that the action of reducing agents naturally occurring in flour could be reversed by oxidation. Further, the naturally occurring proteolytic enzymes of the second clear flour produced no demonstrable irreversible breakdown. In contrast, when papain was added in amounts sufficient to produce the same characteristics as were shown by added reducing agents, the effect was not reversible by subsequent oxidation. In view of these results Sandstedt and Fortmann state:

While proteolysis and reduction produce characteristics in doughs and bread that are visually indistinguishable, they have a decidedly different action on the gluten proteins. Since no perceptible irreversible effect of proteolysis was found in the second clear doughs, we conclude that even the relatively large quantity of naturally occurring proteolytic enzyme present in the second clear flour (as compared to that present in normal patent or straight flours) was present in such small amounts as to be of no significance in baking.

Despite the large volume of work published on the question of the significance of proteinase in panary fermentation and the conflicting nature of much of the evidence, the following conclusions appear justified: (1) Proteinases are present even in patent flours milled from sound wheat. The concentration of such enzymes in normal flours is low—so low that it is difficult to measure their action accurately and reproducibly. (2) Flour proteinases are of the papain type, and can therefore be activated by reducing agents such as those naturally occurring in flour, those occurring in other dough ingredients (notably in yeast), or those added experimentally; moreover, these enzymes may be inactivated by the addition of suitable oxidizing agents such as bromate, chlorite, iodate, or vanadate. (3) Despite the analogy between the effect of oxidizing agents on proteinases and their action as bread improvers, it does not necessarily follow that the primary function of improvers is to inhibit the working of flour proteinases.

The bulk of available evidence indicates that the most probable mechanism for the action of oxidizing agents is that, while they do inactivate proteinases, they also exert an oxidative effect directly on flour protein, and thus change its physical properties. Most of the evidence, though not as precise as could be desired, suggests that the concentration of proteinases in normal flours is so small as to have little effect on gluten properties. For all practical purposes, therefore, the action of oxidizing improvers may be regarded as being a direct one on flour proteins; the undoubted inhibitory action of oxidants on flour proteinases may be considered of little or no significance.

The proteases of flour may have effects in panary fermentation other than a deleterious action on gluten structure. In bucky doughs, limited protein hydrolysis may bring about improved gluten characteristics. Moreover, as yeast is known to utilize nitrogen compounds in its metabolism, it seems possible that some production of such nitrogenous materials by proteolytic action may be desirable in the interest of optimum yeast nutrition.

Read and Haas (1936) demonstrated definitely that the baking quality of certain flours that produce bucky doughs could be improved by the addition of small amounts of plant proteases. Thus, with a number of flours, the loaf volume was increased by 10 to 20 per cent and the texture and grain of the finished loaf benefited materially by the addition of small amounts of malt diastase, taka-diastase, wheat malt flour, papain, or pepsin. Similar improvements were noted when the enzyme preparations were extracted and the proteinase isolated by precipitation with safranine (as suggested by Marston, 1923), and the concentrates added. It was thus evident that the improving action on bucky doughs was brought about by proteases, and not by other enzymes present in the materials added.

That there is an appreciable utilization of nitrogenous material by

yeast during the course of dough fermentation has been pointed out by Amos (see Kent-Jones, 1939, p. 486) and corroborated by Freilich and Frey (1943) and by Shen and Geddes (1942). Typical data, taken from the work of Shen and Geddes, are shown in Table IV.

TABLE IV

EFFECT OF YEAST AND FERMENTATION (OR REST) TIME ON AMINO NITROGEN CONTENT[1] OF DOUGHS MADE WITH LOW-GRADE FLOUR (From Shen and Geddes, 1942)

Fermentation[2] (or rest) time	Basic dough		Basic dough without yeast	
	Without octyl alcohol	With octyl alcohol	Without octyl alcohol	With octyl alcohol
hrs	*mg*	*mg*	*mg*	*mg*
1.5	22.1	28.2	22.1	21.3
3.0	7.1	31.7	24.4	22.3
4.5	6.1	33.5	25.6	22.8

[1] Amino nitrogen per dough weight equivalent to 100 g flour.
[2] Proofing time (55 minutes) is not included in the times record.

It is evident that a large percentage of the amino nitrogen compounds originally present in fermenting doughs disappears during the course of a normal fermentation. It does not follow that proteolysis is required to provide an adequate supply of nitrogenous materials for optimum metabolism; it is entirely possible that there is an adequate supply of such materials existing in flour prior to the onset of enzymatic action. The data of Table IV indicate that, in this particular fermentation, a total of 33.5 mg of amino nitrogen per 100 g flour was present after fermentation for 4.5 hours when yeast activity was inhibited by the addition of octyl alcohol. Some of this nitrogen apparently was supplied by the yeast itself since a similar dough without yeast contained only 22.8 mg amino nitrogen after 4.5 hours. Despite the indication of an extremely slow increase in amino nitrogen from 1.5 to 4.5 hours' fermentation, it appears that a fairly large percentage of the total amino nitrogen existed as such prior to enzymatic action, although it would be expected that the rate of proteolysis would be greatest in the earliest stages of fermentation. During the period covered by these data, yeast apparently utilized 27 mg of amino nitrogen per 100 g flour, which is probably only slightly more than was originally present. It seems doubtful, therefore, that proteolytic action plays a significant part in providing nitrogenous materials for yeast metabolism.

MALTED WHEAT FLOUR

It has been concluded that proteinases are not present in significant amounts in flour milled from normal sound wheat, and it is therefore in

order to consider the possible effect of the addition of products known to be relatively richer in these enzymes. In recent years it has become common practice to add malted wheat or malted wheat flour in order to obtain flours of adequate gas-producing capacity. Such malt products are added primarily as amylase concentrates. However, the process of malting brings about a measurable increase in proteolytic activity. An exhaustive study of the proteinases of malted wheat flour was made by Mounfield (1936, 1938). In 1936, using edestin as a substrate, he showed that sprouted wheat contained a proteinase with a hydrogen-ion optimum of pH 4.1. He reported that, during the process of germination, the proteinase activity of wheat is multiplied approximately six times in 4 days. In 1938, the same author showed that results obtained with edestin could not be applied where either gluten or a mixture of glutenin and gliadin were used. In contrast to results previously reported by the same author using edestin, and to the results of Balls and Hale, no activating effect of cyanide could be found when gluten was used as substrate.

Quantitative data on the proteolytic activity of malted wheat flours are relatively meager. In Tables V and VI are shown typical results taken from studies of Balls and Hale (1936) and Hildebrand (1939).

TABLE V

PROTEASE ACTIVITY OF FLOUR AND MALT (Adapted from Balls and Hale, 1936)

Material	Proteolytic activity[1]		
	Unactivated	Activated by	
		Glutathione	Cysteine
	ml 0.1 *N KOH*	*ml* 0.1 *N KOH*	*ml* 0.1 *N KOH*
Whole wheat flour (a)	1.95	2.40	—
Whole wheat flour (b)	0.70	—	1.15
Wheat malt	3.00	—	3.15
White flour	0.45	—	0.75

[1] Activity expressed as increase in amino nitrogen on autolysis, determined by alcoholic titration.

Since the data were obtained by two different methods they are not strictly comparable, but they serve to show the relative activity of malted wheat and malt flours compared with that of unmalted wheat and flour. The data of Balls and Hale indicate that wheat malt has approximately seven times the proteinase activity of patent flour and from two to three times that of the whole wheat flour. These results were secured by determining the increase in amino groups by alcoholic titration. The data of Table VI, obtained by use of the rate-of-gelation procedure of Landis and Frey (1938), show that malt flour may be from three to twenty times as ac-

tive proteolytically as patent flour, but may be lower in activity than a second clear flour produced from unmalted wheat.

That the proteinase of malted wheat shows somewhat higher activity in the presence of yeast is indicated by the data of Table VII. Presumably, in this case, glutathione or other reducing agents contributed by

TABLE VI

PROTEINASE ACTIVITY OF FLOURS FROM MALTED AND UNMALTED WHEAT (Adapted from Hildebrand, 1939)

Material	Proteolytic activity[1]
	milliunits per g
Patent flour, A mix	0.3
First clear, A mix	0.9
Second clear, A mix	3.6
Patent flour, B mix	0.6
First clear, B mix	1.2
Second clear, B mix	4.8
Malt flour (a)	2.0
Malt flour (b)	6.2
Ground wheat malt	3.9

[1] Activity determined by rate-of-gelation procedure of Landis and Frey (1938).

the yeast bring about the increased activity shown. Where octyl alcohol is used to inhibit yeast action, there is a further increase in apparent activity as measured by increase in nonprotein nitrogen. The difference may be explained by the assumption that some of the nonprotein nitrogenous prod-

TABLE VII

PROTEINASE ACTIVITY OF MALTED AND UNMALTED WHEAT FLOUR (From Hildebrand and Burkert, 1942)

Sample	Proteinase activity (as mg nonprotein N/100 g)		
	No yeast	3% yeast	3% yeast plus octyl alcohol
Patent flour A	117	94	—
Patent flour B	48	70	—
Patent flour C	31	50	
Malt flour A	257	310	348
Malt flour B	233	276	301
Malt flour C	317	336	378

ucts formed as a result of proteolysis are utilized by yeast. The figures of the last column may then be presumed to give a true measure of the proteolytic activity of malt flours in fermenting doughs. The increase in activity amounts to 25 to 50 per cent over that shown by malt flours in the absence of yeast. It should be pointed out that there is reason to believe

that the same type of proteinase activation will occur in fermenting doughs made without malt flour; therefore, the relative concentration of these enzymes in flours from malted and unmalted wheat is probably not greatly different in the presence and in the absence of yeast.

It has been shown repeatedly that the addition of excessive amounts of malted wheat flour to doughs causes increased mobility and, with extended fermentation periods, may give rise to excessive stickiness. Early workers believed that these effects were due entirely to protein hydrolysis, but in 1933 Kozmin advanced the idea that the production of sticky doughs and moist crumb was due to liquefaction and dextrinization of starch. Brief experiments led her to conclude that the hydrolytic changes in gluten structure were not of sufficient magnitude to account for the effects noted. This theory has received increasing support from other investigators in succeeding years. Read and Haas (1936) found that malted wheat flours had a relatively low proteolytic activity compared with that of other proteases, and they therefore concluded that the sticky quality of doughs containing high dosages of malted wheat flour must result from alpha-amylase action. These authors also treated aqueous extracts of various enzymatically active materials with safranine in order to remove proteases. They found that malt preparations thus treated, and hence presumably free from proteases, were still able to cause stickiness in doughs.

Bohn and Bailey (1937), in studying changes in the physical properties of doughs, found that the addition of malted wheat flour appeared to decrease dough elasticity and to increase its mobile properties. Although doughs prepared with 1 and 2 per cent of malted wheat flour were found to be more sticky and mellow than fermented doughs with 0.5 per cent of malt flour, differences in physical behavior were not detectable by either stress or 5-minute farinograph measurements. These authors state:

> It has not been proven that the softening effect is entirely due to the papainase enzyme present in flour. It is possible that other enzymes, proteolytic, diastatic and otherwise, may also be partly responsible for the mellowing effect. It is difficult to obtain pure proteolytic enzymes free from amylases and *vice versa,* and thus the problem as to what enzyme or enzymes cause the softening effect upon the gluten or dough system remains unsolved.

Munz and Bailey (1937) obtained alpha- and beta-amylase preparations from malted wheat flours by the Ohlsson technique. The protease content of these preparations was reduced by precipitation with safranine. Using the farinograph to follow changes in dough consistency, they found that increase in mobility was obtained only in preparations containing alpha-amylase. When this enzyme was inactivated by acid treatment, extracts exerted no significant effect on dough properties.

Sandstedt, Jolitz, and Blish (1939) concluded that stickiness in

doughs is associated with the presence of amylodextrins produced by the action of alpha-amylase; this observation lends further support to the belief that alpha-amylase or some factor associated with it in malted wheat flour is responsible for stickiness.

Geddes, Hildebrand, and Anderson (1941), in a study of the effect of malting conditions on the properties of malted wheat flour, utilized a series of flours from experimental malts. These flours exhibited a fairly wide range of proteolytic activity. However, when the flours were added to a common untreated base flour in amounts sufficient to give constant and adequate gas production, the amount of proteinase activity contributed by the malted wheat flours was a very small fraction of the total activity in the blends. In no case could as much as 5 per cent of the total activity be ascribed to the malted wheat flour. However, in these experiments the malting conditions were not varied as widely as possible, and at the time the proteolytic activity determinations were carried out, the malted wheats had been in storage for an extended period. While it was believed that such storage would not affect relative results, the delay before making activity determinations does cast some doubt on the reliability of the absolute values found.

Results described by the last-named authors were substantiated by Hildebrand and Burkert (1942). Forty-eight flours from as many malts, exhibiting a wide range in proteinase activity, were used to prepare blends with a single untreated base flour, and the dosage of malt flour was so adjusted as to give uniform gas production in all blends. Calculation of the proteinase activity contributed by the malted wheat flours showed that this amount ranged from 0.08 to 4.0 mg of nonprotein nitrogen per 100 g (method of Ayre and Anderson, 1939), whereas the base flour contributed 117 mg per 100 g. Despite the low proteinase content apparently contributed by the malted wheat flour in these blends, baking studies were carried out to determine whether the variation in the proteolytic activity due to the malt flours could be detected. The levels of malt flour employed were such as to produce flours of entirely satisfactory baking performance. At this level of malt flour treatment, no evidence whatever could be found of significant variation in loaf volume, crumb or crust characteristics, or dough handling properties. Accordingly, it was concluded that the amount of proteinase contributed by malt flours is entirely without significance when the malt flour is added in amounts consistent with normal milling and baking practice.

Further evidence substantiating this conclusion was obtained by adding malt flour extracts to a common base flour in amounts equivalent to 1 to 10 per cent of malt flour (together with additional water and 3 per cent yeast). The doughs were mixed in the farinograph, removed, and al-

lowed to ferment for 4 hours at 30°C and then remixed. It was noted that as the amount of malt flour extract was increased there was a progressive increase in stickiness of the doughs, and a progressively greater difference in mobility between the original doughs and those remixed after 4 hours of fermentation. However, when the proteases of the malt flour extract were precipitated by safranine and an amount of the precipitate equivalent to 5 per cent of malted wheat flour was added, there was no significant alteration in dough consistency or stickiness from the characteristics exhibited by the original dough without added malt extract. Hildebrand and Burkert stated: "The increase in stickiness and in dough mobility noted with excessive treatment of malted wheat flour cannot be ascribed to proteolytic activity of such flours, but is due rather to excessive alpha-amylase activity."

YEAST

Early investigations of the proteolytic enzymes of yeast are numerous but inconclusive. Observations were recorded of the liquefying effect of certain yeast cultures on gelatin, but the disagreement between the results of various workers suggests strongly that different yeast types were used and that these varied widely in liquefying activity. The early literature on this subject has been well reviewed by Olsen and Bailey (1925). The most generally held view appears to be that the proteinases of yeast are endocellular—that is, they are formed within the cell and are incapable of diffusing through the cell walls. Olsen and Bailey investigated the effect of the addition of yeast extracts to flours; they followed alterations in protein structure with time by measuring the viscosity of flour suspensions and the soluble nitrogen content. Yeast was cultured in beer wort, and the wort was filtered free of yeast cells. If any proteinases capable of diffusing out of intact yeast cells had been present they would presumably have been extracted by this procedure. As a control, an aliquot portion of the wort was heated to inactivate any enzymes. No difference was found in the characteristics of flour suspensions to which the heated and unheated worts had been added. As a result of these and other studies, Olsen and Bailey state: "These several observations indicate that the proteases contributed by sound, normal intact yeast cells (bakers' yeast) are negligible in their effect upon the properties of gluten during a 4- or 5-hour fermentation period." Since there appear to be no further experimental data to challenge seriously the correctness of this view, it may be concluded that, while proteolytic enzymes are present in intact yeast cells, they have no appreciable effect on gluten proteins during a normal bread-dough fermentation.

If yeast cells are destroyed, the endocellular proteases of these cells are then free to act on the dough proteins. When this occurs, some or all

of the otherwise damaging action can be prevented by the addition of oxidizing agents. For example, army baking for troops overseas is often done using an active dry yeast. On long-continued storage, particularly at elevated temperatures, the dry yeast deteriorates and becomes a source of proteases. Wodicka (1943) discusses this problem and reports the development of a "bread stabilizer" which contains sufficient bromate to inhibit the action of proteinases which may be elaborated by these yeast cells.

INSECT-DAMAGED WHEAT

In one other respect proteolytic activity in wheat is of significance. In Europe, Asia, and North Africa, certain insects of the species *Eurygaster*, *Aelia*, *Dolycoris*, etc., may attack the kernel during the ripening period and lower the viability and baking quality of the grain thus damaged (Berliner, 1931). Studies on this problem from 1931 to 1943 have been reviewed by Kretovich (1944), who carried out a careful investigation of the biochemical changes brought about by the action of these insects. Initial studies of nitrogen fractionation suggested that infestation by the insects had increased the solubility of proteins in both water and 60 per cent alcohol. It was evident that the protein of the grain had undergone a profound change suggesting extensive proteolysis. The extent of such action was measured by determining total water-soluble nitrogen, and the nitrogen not precipitated by 2 per cent trichloroacetic acid, before and after autolysis. Kretovich's data, shown in Table VIII, demonstrate unquestionably that marked proteolysis had occurred as a result of this insect damage.

TABLE VIII

CHANGE IN SOLUBLE NITROGEN FRACTIONS OF NORMAL AND DAMAGED KERNELS DURING AUTOLYSIS (From Kretovich, 1944)

Sample number	Kernels	Nitrogen in per cent of total nitrogen of sample			
		Before autolysis		After autolysis	
		Water-soluble	Not precipitated by CCl_3COOH	Water-soluble	Not precipitated by CCl_3COOH
		%	%	%	%
1	Normal	12.5	7.6	22.7	16.1
	Damaged	22.0	16.3	52.7	43.8
2	Normal	10.0	4.7	21.2	13.4
	Damaged	19.9	14.0	56.9	42.3

This finding was further confirmed by measurements of extensibility of gluten washed from doughs made up with extracts of normal wheat on the one hand and of infected wheat on the other. Extensibility observed in the glutens prepared with extracts of damaged wheat was from 75 to 3000 times greater than that with extracts from normal wheat. The apparent

hydrogen-ion optimum for this type of proteolysis lay between pH 6 and 7. The excessive protein breakdown observed in the presence of damaged wheat could be greatly reduced by moderate acidification. In addition, the quality of the wheat damaged by infestation with these types of insects could be materially improved by heat treatment, either with warm air or with steam.

Insect pests which bring about a type of damage superficially similar to that caused by the *Eurygaster* and *Aelia* species have recently been reported in North America. The green grain bug (*Chlorochroa uhleri* Stål.) and Say's grain bug (*Chlorochroa sayi* Stål.) both attack the immature seed, pierce the outer glume and kernel, and withdraw the liquid contents. The kernel on ripening is shrivelled, grades low, and gives a poor flour yield (Jacobson, 1940, and Harris *et al.*, 1941). No conclusive evidence is available to indicate whether or not these insects, like the European species, inject proteolytically active secretions into the damaged grain. Harris, Sibbitt, Munro, and Telford (1941) studied flour milled from wheat containing varying percentages of kernels damaged by the green grain bug; where damage was severe, doughs from these samples were "short" and "dead." It was impossible to wash gluten from some of the "injured" samples; the gluten disintegrated completely during the washing. This behavior is suggestive of excessive proteolysis; but, since the authors did not demonstrate the presence of excessive proteolytic activity by direct measurement, it cannot be stated definitely that proteolysis is the major factor responsible for the phenomena observed.

Evidence* concerning the nature of the action of Say's grain bug is likewise inconclusive. Some indication of increased proteolytic activity was observed in wheat samples containing 75 per cent of damaged kernels, but, with samples less severely injured, no increase in proteolysis over that of sound grain was found by formol titration or trichloroacetic acid precipitation techniques.

It may be concluded that the injection of a proteolytically active fluid is responsible for much of the damage to baking quality caused by the European grain bugs. But the biochemical changes brought about by attack by *Chlorochroa* in North American grain may or may not be the same; the evidence as yet available is not conclusive.

BIBLIOGRAPHY

AYRE, C. A., and ANDERSON, J. A. (1939). Varietal differences in barleys and malts. VI. Autolytic proteolytic activity of malt and its correlations with wort nitrogen and barley nitrogen fractions. *Can. J. Research* C17:239–246.

* Private communication from J. A. Anderson, Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg, Manitoba.

BAKER, J. C., and MIZE, M. D. (1937). Mixing doughs in vacuum and in the presence of various gases. *Cereal Chem.* 14:721–734.

—— and —— (1939). The relation of mechanical stirring to sponge doughs. *Cereal Chem.* 16:294–295.

—— and —— (1941). The origin of the gas cell in bread dough. *Cereal Chem.* 18: 19–34.

BAKER, J. L., and HULTON, H. F. E. (1908). Considerations affecting the "strength" of wheat flours. *J. Soc. Chem. Ind.* 27:368–376.

BALLAND, A. (1884). Altérations qu'éprouvent les farines en vieillissant. *Ann. chim. phys.* 1:533–554.

BALLS, A. K., and HALE, W. S. (1935). Proteolysis in flours. *J. Assoc. Official Agr. Chem.* 18:135–140.

—— and —— (1936). Proteolytic enzymes of flour. *Cereal Chem.* 13:54–60.

—— and —— (1936a). Further studies on the activity of proteinase in flour. *Cereal Chem.* 13:656–664.

—— and —— (1938). The preparation and properties of wheat proteinase. *Cereal Chem.* 15:622–628.

BERLINER, E. (1931). "Leimkleberweizen" ist "Wanzenweizen." *Mühlenlab.* 1: 25–26.

BOHN, L. J., and BAILEY, C. H. (1937). Effect of fermentation, certain dough ingredients, and proteases upon the physical properties of flour doughs. *Cereal Chem.* 14:335–348.

CAIRNS, A., and BAILEY, C. H. (1928). A study of the proteoclastic activity of flour. *Cereal Chem.* 5:79–104.

ELION, E. (1943). The action of glutathione and wheat germ on dough in relation to proteolytic enzymes in wheat flour. *Cereal Chem.* 20:234–250.

FLOHIL, J. T. (1936). The effect of chemical flour improvers on proteolytic action in relation to the gas-retaining capacity of fermenting doughs. *Cereal Chem.* 13:675–689.

FORD, J. S., and GUTHRIE, J. M. (1908). The amylolytic and proteolytic ferments of wheaten flours, and their relation to "baking strength." *J. Soc. Chem. Ind.* 27:389–393.

FORD, W. P., and MAIDEN, A. M. (1938). Effects in dough of glutathione and papain. *J. Soc. Chem. Ind.* 57:278–281.

FREILICH, J., and FREY, C. N. (1939). Dough oxidation and mixing studies. I. The action of potassium bromate in dough. II. Effects of remixing after fermentation. III. The effects of proteases and reducing substances on dough when mixed in oxygen. *Cereal Chem.* 16:485–512.

—— and —— (1943). Dough oxidation and mixing studies. V. Correlation between protease activity, reducing matter, and oxidizing effects in dough. *Cereal Chem.* 20:301–310.

GEDDES, W. F. (1930). Chemical and physico-chemical changes in wheat and wheat products induced by elevated temperatures. III. The influence of germ constituents on baking quality and their relation to improvement in flour induced by heat and chemical improvers. *Can. J. Research* 2:195–213.

——, HILDEBRAND, F. C., and ANDERSON, J. A. (1941). The effect of wheat type, protein content and malting conditions on the properties of malted wheat flour. *Cereal Chem.* 18:42–60.

HALE, W. S. (1939). The proteinase in wheat flour. *Cereal Chem.* 16:695–702.

HARRIS, R. H., SIBBITT, L. D., MUNRO, J. A., and TELFORD, H. S. (1941). The effect of the green grain bug upon the milling and baking quality of wheat. *Agr. Expt. Sta. N. Dakota Bimonthly Bull.* No. 3:10–14.

HILDEBRAND, F. C. (1939). A comparison of methods for the determination of proteolytic activity. *Cereal Chem.* 16:792–798.

—— and BURKERT, G. M. (1942). Amylase and protease systems of malted wheat flours. *Cereal Chem.* 19:27–35.

JACOBSON, L. A. (**1940**). Say's grain bug in western Canada. *Cargill Crop Bull.* **15**, No. 17:35–38.

JØRGENSEN, H. (**1935**). Ein Beitrag zur Beleuchtung der hemmenden Wirkung von Oxydationsmitteln auf proteolytische Enzymtätigkeit. Über die Natur der Einwirkung von Kaliumbromat und analogen Stoffen auf die Backfähigkeit des Weizenmehles. I. *Biochem. Z.* **280**:1–37.

—— (**1935a**). Ein Beitrag zur Beleuchtung der hemmenden Wirkung von Oxydationsmitteln auf proteolytische Enzymtätigkeit. Über die Natur der Einwirkung von Kaliumbromat und analogen Stoffen auf die Backfähigkeit des Weizenmehles. II. *Biochem. Z.* **283**:134–135.

—— (**1936**). On the existence of powerful but latent proteolytic enzymes in wheat flour. *Cereal Chem.* **13**:346–355.

—— (**1939**). Further investigations into the nature of the action of bromates and ascorbic acid on the baking strength of wheat flour. *Cereal Chem.* **16**:51–60.

KENT-JONES, D. W. (**1939**). *Modern Cereal Chemistry.* 3rd ed. Northern, Liverpool.

KOZMIN, N. (**1933**). Biochemical characteristics of dough and bread from sprouted grain. *Cereal Chem.* **10**:420–436.

KRETOVICH, V. L. (**1944**). Biochemistry of the damage to grain by the wheat-bug. *Cereal Chem.* **21**:1–16.

LANDIS, Q., and FREY, C. N. (**1938**). A sensitive method for the determination of proteolytic activity. *Cereal Chem.* **15**:91–101.

MARSTON, H. R. (**1923**). The azine and azonium compounds of the proteolytic enzymes. I. *Biochem. J.* **17**:851–859.

MOUNFIELD, J. D. (**1936**). The proteolytic enzymes of sprouted wheat. I and II. *Biochem. J.* **30**:549–557, 1778–1786.

—— (**1938**). The proteolytic enzymes of sprouted wheat. III. *Biochem. J.* **32**:1675–1684.

MUNZ, E., and BAILEY, C. H. (**1937**). Effect of the enzymes of malted wheat flour upon certain properties of flour and dough. *Cereal Chem.* **14**:445–457.

OLCOTT, H. S., SAPIRSTEIN, L. A., and BLISH, M. J. (**1943**). Stability of wheat gluten dispersions toward reducing agents in the presence and absence of gluten proteinase. *Cereal Chem.* **20**:87–97.

OLSEN, A. G., and BAILEY, C. H. (**1925**). A study of the proteases of bread yeast. *Cereal Chem.* **2**:68–86.

READ, J. W., and HAAS, L. W. (**1936**). Further studies on the baking quality of flour as affected by certain enzyme actions. Relative starch liquefying power of the diastatic agents investigated. *Cereal Chem.* **13**:14–37.

SANDSTEDT, R. M., and FORTMANN, K. (**1943**). The relation of proteolysis to the characteristics of oxidation and reduction in doughs. *Cereal Chem.* **20**:517–528.

——, JOLITZ, C. E., and BLISH, M. J. (**1939**). Starch in relation to some baking properties of flour. *Cereal Chem.* **16**:780–792.

SHEN, T., and GEDDES, W. F. (**1942**). Protease activity and reducing matter content of wheat flour doughs in relation to baking behaviour. *Cereal Chem.* **19**:609–631.

STOCKHAM, W. L. (**1920**). Some factors related to the quality of wheat and strength of flour. *Agr. Expt. Sta. N. Dakota Bull.* No. 139.

SULLIVAN, B., HOWE, M., SCHMALZ, F. D., and ASTLEFORD, G. R. (**1940**). The action of oxidizing and reducing agents on flour. *Cereal Chem.* **17**:507–528.

SWANSON, C. O., and TAGUE, E. L. (**1916**). A study of certain conditions which affect the activity of proteolytic enzymes in wheat flour. *J. Am. Chem. Soc.* **38**:1098–1109.

WODICKA, V. O. (**1943**). Bread stabilizers for use with active dry yeast. *U. S. Army Quartermaster Corps Subsistence Research and Development Lab., Research Repts.* 73–43 (Nov. 2, 1943), 4 pp.

CHAPTER X

THE MECHANISM OF ALCOHOLIC FERMENTATION

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INTRODUCTION

Alcoholic fermentation may be defined as the enzymatic conversion of carbohydrate into ethanol and carbon dioxide with small amounts of glycerol and traces of other products. It should be pointed out that the term applies in a practical sense to the conversion of starches (after saccharification) or sugars into alcohol and carbon dioxide by *Saccharomyces cerevisiae*, commonly known as yeast. Alcoholic fermentation can be brought about, however, by microorganisms other than *Saccharomyces cerevisiae*. These include other species of yeasts and certain genera of bacteria and fungi; in fact, a great many species of microorganisms form substantial quantities of ethanol along with large quantities of other products. Thus such an organism as *Escherichia coli*, commonly found in the intestines of animals, while forming lactic acid, acetic acid, formic acid, carbon dioxide, and hydrogen from glucose, also forms ethanol in relatively large quantities. However, *Sarcina ventriculi*, a species of bacteria, apparently has a metabolism much like that of *Saccharomyces cerevisiae* since it converts glucose into ethanol and carbon dioxide with yields comparable to those of yeast. Certain fungi, *e.g.*, species of *Fusarium*, also produce large yields of ethanol and carbon dioxide, and may be considered to possess an alcoholic type of fermentation.

INTERMEDIARY MECHANISM OF ALCOHOLIC FERMENTATION

There is much evidence to support the view that the basic mechanism of alcoholic fermentation is essentially the same as that which underlies all anaerobic cellular metabolism. In fact, there is reasonable evidence to

support the concept that the underlying mechanism of cellular metabolism is basically similar in green plants, animals, bacteria, yeasts, and fungi, and that this similarity occurs between cells of different tissues of the same organism, *e.g.*, brain, liver, muscle. This relationship would imply that Nature in her evolution has followed a basic pattern, to deviate only in less fundamental behavior from species to species. Thus the final products of the dissimilation of *Saccharomyces cerevisiae* are mainly ethanol and carbon dioxide; of *Aerobacter aerogenes*, acetic, formic, lactic, and succinic acids, 2,3-butylene glycol, acetylmethylcarbinol, ethanol, carbon dioxide, and hydrogen; of the propionic acid bacteria, propionic, succinic, and acetic acids, and carbon dioxide. The mechanisms of anaerobic dissimilation of the three species of microorganisms are, however, fundamentally alike (Werkman, 1939; Werkman and Wood, 1942); only the terminal transformations are different. This concept may be illustrated by Figure 1, which shows the common conversion of carbohydrate to the pyruvate–lactate equilibrium. Beyond this point the dissimilation varies with the

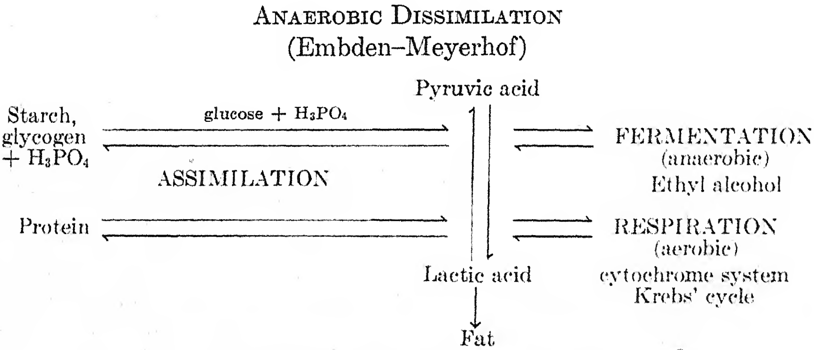


Fig. 1.—Outline of cellular metabolism.

species, and within the species with the environment. Pyruvate may be considered to be the cardinal intermediate of metabolism in that it terminates the common phase and initiates terminal phases of anaerobic dissimilation, which vary with species and environment. Moreover, pyruvate undergoes transamination with the formation of amino acids; it is the product oxidized by oxygen in respiration through the Krebs' cycle and the cytochrome oxidase system; and it may also lead to fat formation. Pyruvate thus appears to be a junction point of the various phases of basic metabolism.

Alcoholic fermentation is essentially an anaerobic process. In fact, the term fermentation generally is used in a scientific sense to refer to anaerobic metabolism, although it is used as a general term, especially in

industry, to refer to any process brought about by microorganisms or enzymes. Initial progress in understanding the phenomena of fermentation was conditioned upon the overthrow of the theory of spontaneous generation. Vallery-Radot (1937) in *The Life of Pasteur* remarks, in speaking of fermentation, that all was darkness, pierced in 1836 by a momentary ray of light. In that year the physicist, Cagniard-Latour, studying the ferment of beer, called yeast, observed that it was composed of cells, "susceptible of reproduction" by a sort of budding, that probably acted on sugar through some effect of their vegetation. In 1837 Schwann (1837, 1839) mentioned alcoholic fermentation as evidence that such changes are brought about only by a living cell.

The classical investigations of Pasteur, which culminated in acceptance of the principle that fermentation is caused by a living cell, began in 1857 with his studies on yeast. He discovered the existence of organisms which do not require atmospheric oxygen and referred to the phenomenon by his famous statement, "*La fermentation est la vie sans air.*" Pasteur considered such a process as an intramolecular oxidation in which part of the glucose is oxidized, whereas another part is reduced. The production of the ethanol and carbon dioxide is anaerobic and requires no participation of atmospheric oxygen. Yeast does possess the aerobic (respiratory) mechanism, and, when grown in the presence of air, as in the industrial production of yeast cells, uses its aerobic system to build cell protoplasm but little alcohol. It is the anaerobic fermentation process that will be discussed in this chapter.

The basic scheme of alcoholic fermentation generally accepted at the present time is referred to as the Embden–Meyerhof scheme (Fig. 1). Glycolysis is the process by which carbohydrates including glycogen, glucose, levulose, or mannose are metabolized to pyruvic acid (or lactic acid). Although Meyerhof has contributed much to the knowledge of carbohydrate breakdown in muscle and yeast cells, the scheme as now widely accepted is a development of a number of earlier workers, particularly Harden, Neuberg, Kostytschew, Neubauer, Lebedew, and von Euler. In more recent years, workers in the laboratories of Cori, Parnas, and Warburg have made important contributions, and these will be referred to in the present discussion only as they are directly involved in the formulation of the presently accepted scheme.

The entire scheme may be summarized as a method used by nature to dissimilate carbohydrates to provide energy, necessary to the life and reproduction of the organism, by a series of electron transfers generally, although not always, made apparent by a simultaneous hydrogen transfer. These transfers are made possible by a series of graded electron mediators or transporters. Three coenzymes play important roles: (1) coenzyme I,

(2) adenylic acid, and (3) cocarboxylase. Harden discovered the occurrence of the first two during his studies on alcoholic fermentation. Coenzyme I functions as a hydrogen mediator; the adenylic acid system is necessary in phosphate transfer; but the manner of action of cocarboxylase is not yet clear, although it may serve as an electron mediator. The energy accruing as a result of electron transfer is concentrated in energy-rich phosphate bonds. Reversibility of the entire scheme is initiated by phosphorylation of the carbohydrate substrate.

KINETICS OF ALCOHOLIC FERMENTATION

The early literature of alcoholic fermentation has been adequately reviewed by Harden (1932). For present purposes, the discussion may be limited largely to the modern concepts dating from 1933, the time of the inception of the present views that culminated in the generally accepted outline known as the Embden–Meyerhof scheme of glycolysis. True it is that present ideas have their roots in the basic work done prior to 1933 by such pioneers as Harden, Neuberg, Meyerhof, Neubauer, Magnus-Levy, Kostytschew, and Kluyver, among others; nevertheless 1933 forms a natural point in history to initiate a discussion of our present views. Even in so short a period in scientific development, many basic contributions have been made to the original scheme of Embden (1933) and of Meyerhof (1942). The laboratories of Meyerhof, Warburg, Parnas, von Euler, and Cori have done most to bring the scheme to its present stage of completion.

In 1933, Embden, Deuticke, and Kraft (1933), and independently Meyerhof and Kiessling (1933), proposed a modification of the then generally accepted scheme of Neuberg, that eliminated methylglyoxal as an intermediate entirely and replaced it by phosphoglyceric acid. Moreover, the roles of hexose monophosphate and hexose diphosphate were clarified; and this has since resulted in conciliating the two schools of thought, the "monophosphaters" and the "diphosphaters," each of which believed in the cardinal importance of its respective sugar–phosphate ester in alcoholic fermentation. The series of reactions now widely accepted is shown in Figure 2 and is discussed in detail in the following paragraphs.

Within the cell, the initial stage of glycolysis is a phosphorylation of glucose by phosphorolysis, *i.e.*, splitting of glycogen or starch by phosphoric acid with formation of glucose-1-phosphate, the Cori ester. This initial phosphorolysis was discovered by Parnas and Baranowski (1935, 1937), and successfully studied by Cori and by Kiessling and Schäffner, among others. Though the reaction is reversible, glucose-1-phosphate is not converted into polysaccharide unless a catalytic quantity of polysac-

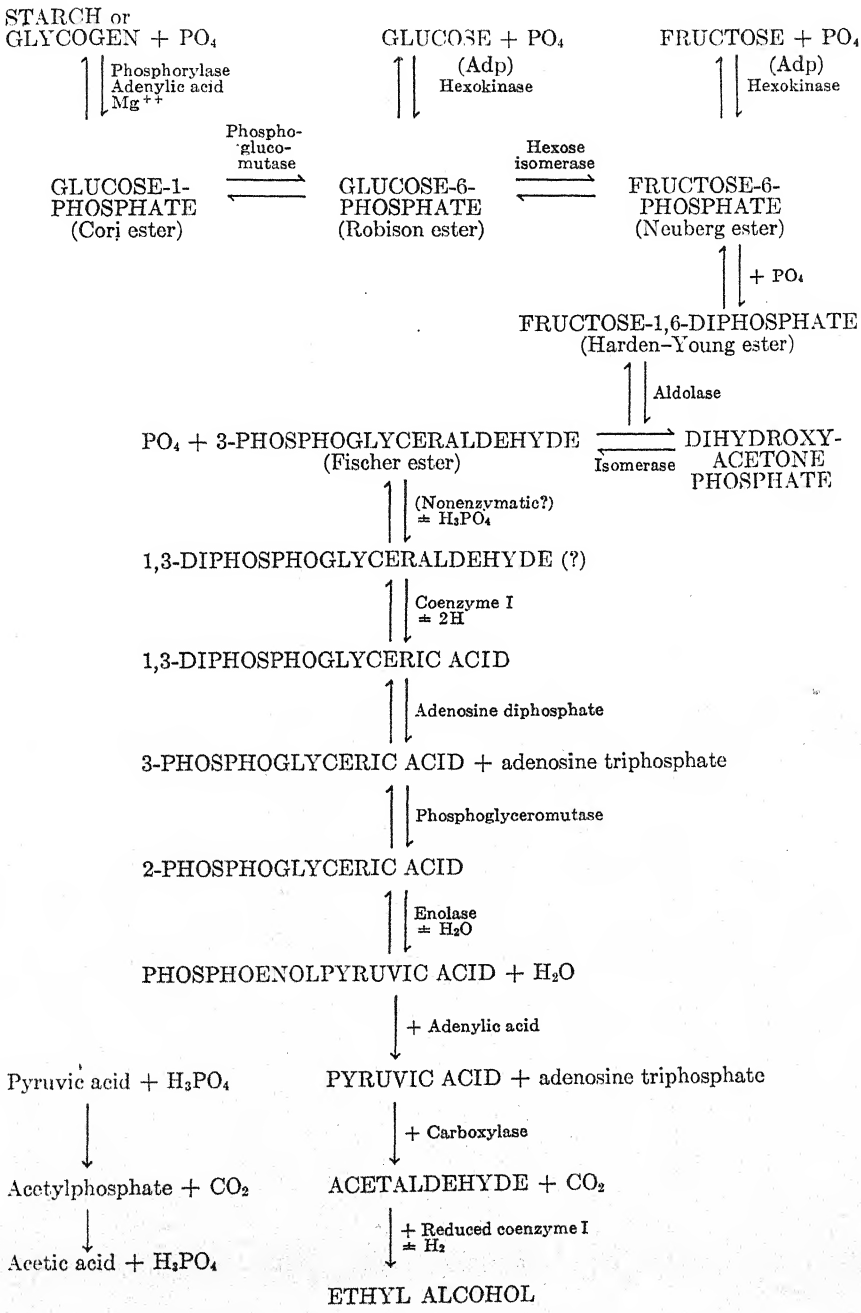


Fig. 2.—Scheme of alcoholic fermentation.

charide is present (Adair, 1928; Bear and Cori, 1941). At pH 7.0 the equilibrium is 77:23 and at pH 5 it is 92:8 in favor of glycogen; the enzyme is known as phosphorylase and is a protein–magnesium–adenylic acid complex. Nilsson and Alm (1936, 1940), however, maintain that fermentation reactions in yeast cells may proceed without the intervention of phosphorylation in the early stage. They found that dried yeast cells could ferment glucose in the absence of phosphate with the formation of a nonfermentable compound containing three carbon atoms.

Phosphorylases are found in muscle, animal organs, bacteria, and typical plants, as well as in yeast; the potato is an especially satisfactory source (Green and Stumpf, 1941; Hanes, 1940). Cori and co-workers (1939), who prepared phosphorylase from various animal organs and muscle, believe that adenylic acid in trace concentration is required as a coenzyme (1938). Kiessling (1939) does not believe adenylic acid is necessary. Apparently it is not required as a coenzyme by potato phosphorylase, or traces of adenylic acid may be firmly bound to the enzyme. Both plant and animal phosphorylases are activated by reducing agents but glucose inhibits their action (Cori and Cori, 1940; Hanes, 1940). For methods of preparing phosphorylase consult Sumner and Somers (1943).

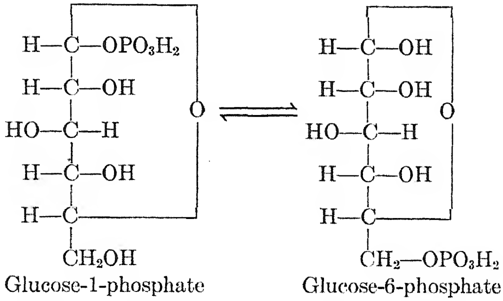
Glycogen and starch contain both the 1,6 as well as the 1,4 linkage, and phosphorylase may be specific for one or the other bond. Potato phosphorylase attacks only the 1,4 connection (Meyer and Bernfeld, 1942), whereas yeast phosphorylase, as now prepared, attacks both bonds and is a mixture of both types of phosphorylase. Muscle also contains both types.

The Cori ester, glucose-1-phosphate, formed by the initial phosphorolysis of starch or glycogen (Cori *et al.*, 1936, 1937, 1938; Cori, 1942) is rearranged in the yeast cell into glucose-6-phosphate, the Robison ester. Phosphoglucomutase (Cori *et al.*, 1938, 1940; Sutherland *et al.*, 1941) a protein–magnesium enzyme, catalyzes the reaction, and the equilibrium yields a ratio of 6:94 of the Cori and Robison esters, respectively.

Robison (1922) first isolated hexose monophosphate and showed it to consist of a mixture of 60 per cent glucose-6-phosphate and 40 per cent fructose-6-phosphate. Juices and dried yeast preparations (*e.g.*, Lebedew) usually accumulate fructose-1-6-diphosphate; however, Kluyver and Struyk (1928) showed that the ratio of the diester to the monoester which accumulates can be varied to favor the latter by dilution.

The divalent magnesium ion may be replaced by the divalent cobalt or manganese ions. The enzyme has been obtained free from phosphorylase and phosphohexose isomerase by Sutherland *et al.* (1941). Optimal hydrogen-ion concentration is from pH 7.5 to 9.2 (Sumner and Somers, 1943).

The action of phosphoglucomutase may be illustrated:



The extracellular attack on starch or glycogen is initiated by a path entirely different from that used in the internal metabolism of the cell. Extracellular fermentation of starch or glycogen starts with a hydrolysis brought about by amylases or glucosidases. In the industrial production of ethanol, the raw material for conversion is necessarily a simple carbohydrate, since yeast does not excrete the necessary amylase to attack starch. Usually blackstrap molasses or a hydrolyzed starch (corn or wheat) is employed; occasionally some other raw material is used because of local supply, *e.g.*, fruit juices, wood sugar. The complete fermentation then involves the conversion of glucose, or a mixture of glucose and fructose, into alcohol and carbon dioxide.

The phosphorylation of the glucose to glucose-6-phosphate is brought about by hexokinase; levulose or mannose is likewise phosphorylated to the 6-phosphate. In this phosphorylation the energy-rich adenosine triphosphate (Fig. 3) functions as a coenzyme.

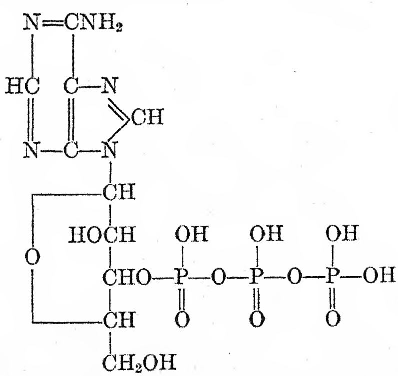


Fig. 3.—Adenosine triphosphate from yeast. Muscle adenylic acid differs from yeast adenylic acid in having the phosphate attached to position 5 of the ribose.

Colowick and Kalckar (1941) found the reaction with yeast hexokinase to be:

$$\text{adenosine triphosphate} + \text{glucose} \longrightarrow \text{adenosine diphosphate} + \text{glucose-6-phosphate}$$

When myokinase, a heat stable protein, was added to the hexokinase, the adenosine diphosphate transferred one phosphate to glucose. The reaction, however, is not simple (Kalckar, 1942) but probably involves a "dismutation" of adenosine diphosphate, thus

$$\text{2 adenosine diphosphate} \longrightarrow \text{adenosine triphosphate} + \text{adenylic acid}$$

Meyerhof (1935) had described the over-all reaction in 1935.

Lohmann (1933) found an enzyme present in muscle extracts which catalyzed a reversible transformation of glucose-6-phosphate into fructose-6-phosphate. The enzyme is called phosphohexoisomerase. Later it was shown to be present in yeast and higher plants (Hanes, 1940). The equilibrium is 70:30 in favor of glucose-6-phosphate.

Fructose-6-phosphate is converted into fructose-1,6-diphosphate by phosphohexokinase. Adenosine triphosphate again provides the additional phosphate group, and is dephosphorylated to adenosine diphosphate (Ostern, Guthke, and Terszakowec, 1936).

Introduction of the second phosphate group requires an energy-rich group (Meyerhof *et al.*, 1937, 1938). However, Lipmann (1941) from Lundsgaard's results, suggests a more efficient process in the living cell, *i.e.*,

$$\text{2 hexose monophosphate} \longrightarrow \text{1 hexose diphosphate} + \text{1 hexose}$$

Since interchange of ester phosphate is known to occur, glucose-1-phosphate might well transfer its phosphate into the 1 position of fructose-6-phosphate. Thus no energy-rich phosphate bond would be sacrificed to force the second group into the monophosphate in contrast to the fermentation of glucose in which both phosphate groups must be forced in by the sacrifice of two energy-rich phosphate bonds.

The enzyme system has not received adequate study; it has been considered irreversible (Kalckar, 1941). Cori (1939), however, presents evidence to the contrary. Needham and Pillai (1938) found no transfer of phosphate from fructose diphosphate to adenylic acid.

It may be noted in passing that the triphosphate is restored in later reactions:

$$\text{1,3-diphosphoglyceric acid} + \text{adenosine diphosphate} \rightleftharpoons \text{3-phosphoglyceric acid} + \text{adenosine triphosphate}$$

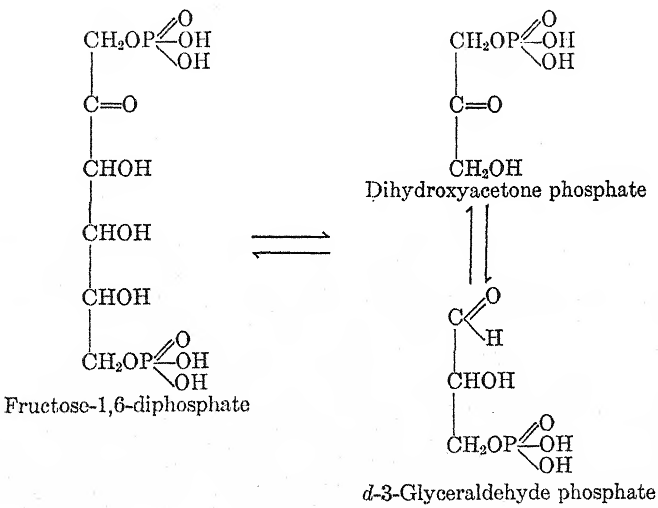
and

$$\text{phosphoenolpyruvic acid} + \text{adenosine diphosphate} \rightleftharpoons \text{pyruvic acid} + \text{adenosine triphosphate}$$

When Harden and Young (1908; see also Young, 1909; Harden, 1932) isolated fructose-1,6-diphosphate from fermenting press juice of yeast, Harden himself did not accept the ester as a true intermediate in

yeast fermentation because it accumulated in an amount molar equivalent to the glucose fermented. He believed that the diphosphate would have to be dephosphorylated to fructose by phosphatase before it could ferment (Harden, 1932).

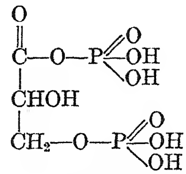
Fructose-1,6-diphosphate is split by aldolase into two trioses, dihydroxyacetone phosphate and 3-phosphoglyceraldehyde (Meyerhof and Lohmann, 1934; Meyerhof, 1937). The trioses are in turn in equilibrium with each other by virtue of triose isomerase. The two equilibrium reactions may be referred to as the zymohexase reaction, a name suggested before the nature of the reaction became clearly known (Meyerhof and Lohmann, 1934, 1934a,b, 1935; Meyerhof *et al.*, 1936; Herbert, Gordon, and Subrahmanyan, 1940), *i.e.*, distribution of the hexose diphosphate into a 50–50 mixture of *l*-dihydroxyacetone phosphate and *d*-3-phosphoglyceraldehyde. The ketotriose is present in a ratio of 95 to 5 of the aldotriose.



However, it is the aldotriose, the *d*-compound, that is converted into phosphoglyceric acid as shown by the formation of *d*-phosphoglyceric acid, which could not be formed from the *l*-compound (Kiessling and Schuster, 1938). Embden, Deuticke, and Kraft (1933) had proposed the occurrence of the zymohexase reaction and the formation of phosphoglyceric acid from phosphoglyceraldehyde on theoretical grounds.

After glycolysis in subsequent reactions has formed one molecule of acetaldehyde (*i.e.*, after the "initial" state of Meyerhof), the entire pathway of sugar breakdown is through 3-phosphoglyceraldehyde which is oxidized in a reaction involving the simultaneous reduction of coenzyme I; the reduced coenzyme I is then reoxidized by acetaldehyde. The alternate

pathway (not shown in Fig. 2), involving the reduction of dihydroxyacetone phosphate to α-glycerophosphate with simultaneous oxidation of reduced coenzyme I, is not followed because acetaldehyde is much the better hydrogen acceptor.



1,3-Diphosphoglyceric acid (Negelein and Brömel, 1939, 1939a)

The 3-phosphoglyceraldehyde is oxidized to 3-phosphoglyceric acid in a reaction coupled with phosphorylation to the 1,3-diphosphoglyceric acid (Warburg and Christian, 1939; Negelein and Brömel, 1939, 1939a).

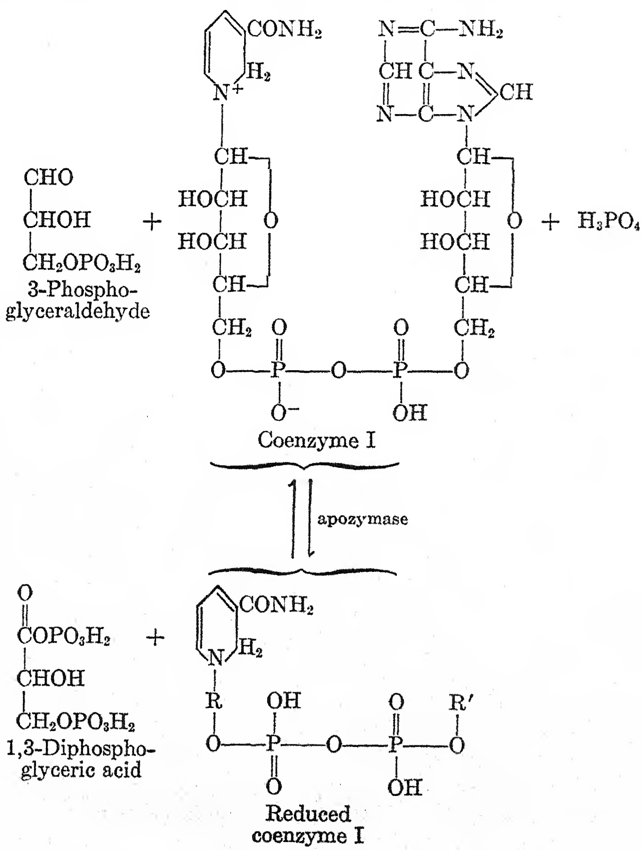


Fig. 4.—Oxidation of 3-phosphoglyceraldehyde.

This is the first oxidation, and the electron (or $H^+ + e$) transfer is mediated through the nicotinamide-containing coenzyme I (cozymase) (Schlenk and Euler, 1936). The coupled reaction (Schlenk, 1942) is shown in Figure 4. Coenzyme I is regenerated by a subsequent transfer of two atoms of hydrogen to acetaldehyde formed later in the scheme by decarboxylation of pyruvic acid. Lipmann (1941) points out an important result of the coupling of the oxidation of phosphoglyceraldehyde and its phosphorylation in that phosphorylation of a carboxyl group makes it more readily reduced. This is important in the synthesis of carbohydrates from compounds more oxidized than phosphoglyceraldehyde.

Coenzyme I is also referred to as codehydrogenase I, cozymase, coferment I, factor V, coreductase, or diphosphopyridine nucleotide. It is found in all plants, animals, and microorganisms, metabolizing carbohydrates. Yeast cells are a particularly rich source and contain approximately 0.5 g per kg (Meyerhof and Ohlmeyer, 1937). From 35 to 45 per cent of the total nucleotide is present as the dihydro form (Ochoa and Ochoa, 1937; Ochoa and Peters, 1938). Coenzyme I acts as a mediator in several reactions, functioning with a specific protein for each substrate. The coenzyme apparently requires different apoenzymes (protein fraction) when functioning in an oxidizing or reducing capacity. The reactions catalyzed are practically all reversible, but equilibrium is not reached under normal conditions because removal of the products maintains a constant flow.

The 1,3-diphosphoglyceric acid is dephosphorylated to 3-phosphoglyceric acid by adenosine diphosphate (Negelein and Brömel, 1939; Warburg *et al.*, 1930, 1939). The reaction may be written:

$$\text{3-phosphoglyceraldehyde} + H_3PO_4 + \text{coenzyme I} + \text{adenosine diphosphate} \rightleftharpoons$$

$$\text{3-phosphoglyceric acid} + \text{reduced coenzyme I} + \text{adenosine triphosphate}$$

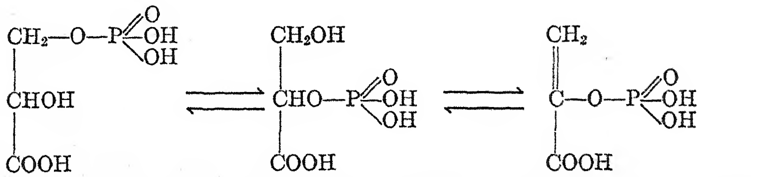
The reduced coenzyme I is oxidized by subsequently formed acetaldehyde which is reduced to form a final product of fermentation, ethyl alcohol. The adenosine triphosphate serves to carry phosphate to glucose.

The oxidation of 3-phosphoglyceraldehyde has been elucidated recently by the work of Warburg and Christian (1939, 1939a) and Negelein and Brömel (1939, 1939a) who showed that the reaction occurs in two steps; recently, Meyerhof and Junowicz-Kocholaty further studied the reaction (see page 307 for discussion). Warburg and Christian considered the formation of the 1,3-diphosphoglyceraldehyde as nonenzymatic. Meyerhof (1941) indicates that the reaction may be enzymatic. Negelein and Brömel (1939a) isolated the 1,3-diphosphoglyceric acid.

Needham and Pillai (1938) found that the oxidation of triose phosphate in the presence of arsenate (replacing phosphate) was not coupled

with the esterification of phosphate. Warburg and Christian offered the explanation that arsenate forms an intermediate with phosphoglyceraldehyde (1-arseno-3-phosphoglyceraldehyde) whose oxidation does not require an (arsenate) acceptor as does 1,3-diphosphoglyceric acid, but which decomposes spontaneously in aqueous solution. Although the 1,3-diphosphoglyceric acid is also unstable, it requires an acceptor, its rate of decomposition being too slow to be of biological significance.

3-Phosphoglyceric acid is in equilibrium with 2-phosphoglyceric acid; the enzyme involved is phosphoglyceromutase (Meyerhof and Kiessling, 1934). Enolase, a magnesium–protein enzyme, converts 2-phosphoglyceric acid to phosphoenolpyruvic acid; this is the reaction referred to in the discussion of energy-rich bonds; the two constituents contain the same total energy value but the energy is concentrated in the phosphate bond in the phospho*enol*pyruvate (Lohmann and Meyerhof, 1934; Pillai, 1938; Kalckar, 1941; Lipmann, 1941):



3-Phosphoglyceric acid 2-Phosphoglyceric acid Phospho*enol*pyruvic acid

By the action of phosphoglyceromutase and enolase an equilibrium occurs with the following composition:

3-phosphoglyceric acid (58.5%) $\rightleftharpoons$ 2-phosphoglyceric acid (12.5%)
$\rightleftharpoons$ phospho*enol*pyruvic acid (29%)

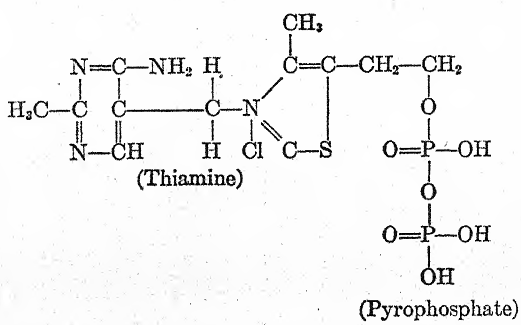
The breakdown of the 2-phosphoglyceric acid may be prevented, with consequent inhibition of the fermentation by adding sodium fluoride which interferes with enolase (Warburg and Christian, 1941; Utter and Werkman, 1942). The magnesium ion is required by enolase under natural conditions but manganese may be substituted. With magnesium the inhibition results from the formation of an insoluble magnesium complex with fluoride; Warburg and Christian (1941) found that a protein–magnesium–fluorophosphate was formed. If manganese is used, sodium fluoride is ineffective in inhibiting enolase. Manganese apparently does not form an insoluble compound, and sufficient manganese remains available to form enolase (Utter and Werkman, 1942).

Phosphopyruvate phosphatase (protein–magnesium–adenosine diphosphate) dephosphorylates the phosphopyruvic acid with the production of pyruvic acid and the restoration of the adenosine triphosphate. The

dephosphorylation normally proceeds in the presence of phosphate acceptors such as glucose and glucose-6-phosphate. In the absence of these acceptors, the reactions may occur in the presence of adenylic acid to form adenosine triphosphate (Parnas, 1936). Pillai (1938) has shown that dephosphorylation of the phosphopyruvic acid may occur in the absence of adenylic acid, glucose, or the hexose monophosphate. This dephosphorylation has been explained by Meyerhof and Junowicz-Kocholaty (1942) as due to the phosphorylation of 3-phosphoglyceric acid to form 1,3-phosphoglyceric acid, which in turn is dephosphorylated to inorganic phosphate. This reaction occurs only when the 3-phosphoglyceric acid is added in relatively high concentration, and has no physiological importance.

The reverse process is generally considered as not occurring and is the only reaction from starch or glucose that is irreversible.

Pyruvic acid is the cardinal and common intermediate of cellular metabolism; in alcoholic fermentation it is decarboxylated to acetaldehyde and carbon dioxide by carboxylase. This enzyme has a coenzyme fraction known as cocarboxylase, a thiamine diphosphate. Lohmann and Schuster (1937) isolated crystalline cocarboxylase from yeast and proved its structure as the pyrophosphoric acid ester of thiamine (vitamin B_1). Thiamine apparently is phosphorylated by adenosine triphosphate to form cocarboxylase (Lohmann and Schuster, 1937). A metal ion such as magnesium or manganese is required by the enzyme, which has been isolated in a highly purified form from yeast by Green, Herbert, and Subrahmanyan (1940, 1941). They believe that the divalent metal acts as a "cement" to bind the apoenzyme to the coenzyme; magnesium or manganese could be replaced by other divalent metals, but not by any of the monovalent or trivalent ones tested. Carboxylase is a firmly bound conjugated protein in high salt concentration but dissociates in alkaline or dilute salt concentration. The chemical constitution of crystalline cocarboxylase is:



Cocarboxylase

Yeast cells contain a phosphatase which liberates free thiamine from cocarboxylase (Melnick and Field, 1939), but other phosphatases liberate only one of the two phosphate groups (Lohmann and Schuster, 1937; Tauber, 1938). Weil-Malherbe (1939) showed that yeast is also able to transfer phosphate from phosphopyruvic acid to thiamine through the adenylic acid system.

The biological effect of thiamine results from the action of its pyrophosphoric acid form. The stimulating effect of thiamine on alcoholic fermentation is used as a standard test for the quantitative determination of thiamine present in various foodstuffs (Schultz, Atkin, and Frey, 1937, 1938). Yeast is also used to determine cocarboxylase quantitatively by the quantity of carbon dioxide liberated from pyruvic acid (Ochoa and Peters, 1938).

Acetaldehyde formed by the decarboxylation of pyruvic acid is reduced to alcohol by the hydrogen released in the dehydrogenation of 1,3-diphosphoglyceraldehyde to 1,3-diphosphoglyceric acid. The hydrogen is transferred to coenzyme I. This is the concluding step in the production of alcohol from starch or glucose.

Alternate end-products can also be obtained. Should the reduction of the acetaldehyde be prevented in some manner, it is possible that another hydrogen acceptor will function, such as glyceraldehyde phosphate (or dihydroxyacetone phosphate). Glycerophosphate is formed by the reduction, and can then be hydrolyzed to glycerol and phosphoric acid by phosphatase. In this case much less ethanol is formed, but relatively large quantities of glycerol are produced. Acetaldehyde may be prevented from acting as a hydrogen acceptor by sulfites which form addition compounds with it. This process has industrial application and the fermentation is known as Neuberg's type 2.

Somewhat similar is Neuberg's type 3 fermentation, which occurs when the reaction is alkaline. In this case, however, the acetaldehyde undergoes dismutation in which the first molecule is oxidized to acetic acid at the expense of the second molecule, which is reduced to ethanol. Hydrogen arising from the oxidation of one molecule of glyceraldehyde phosphate is available to reduce another molecule to glycerophosphate which is hydrolyzed to glycerol and phosphoric acid.

PHOSPHORYLATION

During the development of our knowledge of fermentation, there have been persistent attempts to prove that pathways of glycolysis exist which do not involve phosphorylation. Although there is no conclusive proof that such a pathway (or partial pathway) does not exist, the evidence

is strongly opposed to such a concept. The evidence for nonphosphorylation is largely negative. No completed scheme has been presented in contrast to the precision and nicety with which the phosphorylative scheme has been formulated. Opposing data have been concerned with objections to phosphorylation rather than in support of an alternate scheme. Much of the opposing evidence has come from investigations carried out on tumor tissue, which purports to show that such tissues are unable to utilize phosphorylated hexoses at a rate comparable to hexoses (Potter, 1944) and that glycolysis in the presence of both glucose and hexose diphosphate occurs at a greater rate than with either alone. Yet Boyland and Boyland (1938) have concluded that intermediary glycolysis in tumor tissue was exactly analogous to that of muscle. They showed that tumor tissue rapidly destroyed coenzyme I and adenylic acid, and therefore both had to be added to obtain normal results. Ochoa (1941) demonstrated the need for acceptors of phosphate with brain tissue extracts. Glucose can be metabolized more rapidly than hexose diphosphate when the presence of phosphate acceptors is critically low because glucose functions as an acceptor.

Although most studies on phosphorylation have been carried out on cell-free juices or extracts which form abnormal accumulations of phosphate esters, there is every reason to believe that these accumulations are the result of disturbing the normal delicate balance of enzymes occurring within the intact cell. Some accumulation has been demonstrated to occur within the normal intact cell under these conditions. McFarlane (1937) and Wiggert and Werkman (1930) have demonstrated this for yeast and bacteria, respectively.

Objection is sometimes raised to phosphorylation on the ground that glucose may be utilized by cells, whereas the phosphate esters are not. Frequently the explanation involves permeability of the cell wall; again, glucose may be dissimilated more rapidly in the absence of phosphate acceptors since the glucose can function as such.

The phosphate effect observed by Nilsson and Alm (1936) has been interpreted to mean that phosphorylation does not occur in the living cell. Nilsson and Alm did not so interpret their results, but did assume that sugar was monophosphorylated and the ester then split into two trioses, one of which was phosphorylated. The unphosphorylated triose was assumed to be the one fermented.

Kalckar (1941) offers an explanation which conciliates the views of Nilsson with modern concepts of carbohydrate oxidation. Nilsson and Alm point out that the difference between their quick-dried yeast and slow-dried Lebedew yeast lay in the presence of a phosphatase in their preparation. High sensitivity to phosphate and thermolability identify it

as adenyl pyrophosphatase. It is apparent that the large accumulation of phosphate esters in juice fermentations is an artifact caused by the absence or failure of certain enzymes to function. The absence of adenylpyrophosphatase in yeast juice means that the only way in which adenyl pyrophosphate can be dephosphorylated is in the presence of a phosphate acceptor, *e.g.*, glucose. This necessity for phosphate acceptors has been thoroughly shown by Meyerhof, whose work has been of particular importance in explaining the more rapid fermentation of unphosphorylated sugars under certain conditions. Meyerhof (1937) showed that the absence of a phosphate acceptor accounts for the failure of the preparation of Warburg and Christian to ferment fructose 1,6-diphosphate.

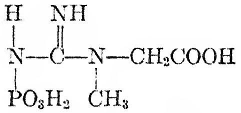
For some thirty years after its discovery, phosphorylation was thought to be a process, the sole purpose of which was to prepare the carbohydrate molecule for splitting into two fragments with three carbon atoms. It is now known that phosphorylation of the carbohydrate molecule initiates a series of reversible reactions in which ester linkages are formed; the phosphate is linked through an alcoholic hydroxyl group as, for example, in hexose phosphates, triose phosphates, glycerophosphates, and 3- or 2-phosphoglyceric acids. Lipmann (1941) refers to this group of phosphorylated compounds formed by ester linkages as energy-low, whereas a smaller group exists in which the phosphate linkage is energy-rich. In many respects the phosphate esters behave like the esters of alcohols and organic acids, and the change in free energy may be calculated from the relationship to the equilibrium constant (Lewis and Randall):

$$\Delta F^0 = -RT \ln K = -4.58 \log K$$

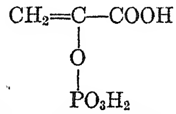
Since the Cori ester, glucose-1-phosphate, occurs in equilibrium with glycogen and inorganic phosphate (Cori, 1939), glucose-1-phosphate $\rightleftharpoons$ glycogen + phosphate, the ester linkage is substantially equivalent to the glucosidic glucose linkage. According to Lipmann (1941), ΔF equals about -3000 cal; he suggests a range of 2000 to 4000 cal to apply to the phosphate group when esterified with an alcoholic hydroxyl group. This group of phospho-organic compounds with ester linkages is the low-potential or energy-poor phosphate bond group. Such substances are relatively stable and only small amounts of energy are involved when the phosphate bonds are broken. The linkages designed to transfer groups with loss of energy (energy-rich bonds) are the weak linkages of chemical nomenclature, the tendency to break the bond is great, and large amounts of energy are available, thus $-\Delta F$ is large. It is the amount of energy made available to do biological work which determines the group potential, or the escaping tendency of the phosphate group.

The energy-rich group of phosphate compounds of biological interest

includes phosphopyruvic acid, adenosine triphosphate and creatine phosphate, and each has a different type of linkage, C—O—P, P—O—P, and N—P, respectively. The fact that a reversible exchange of the phosphate group occurs among members of this group indicates the uniformity of the group potentials of the members. The heat content of the linkages is fairly uniform (Meyerhof and Schulz, 1935) although heat values, ΔH, may lead to erroneous conclusions as to free energy values since the entropy factor unpredictably affects the free energy value. Lipmann (1941) presents evidence for the equivalence of ΔH and ΔF in the present case.

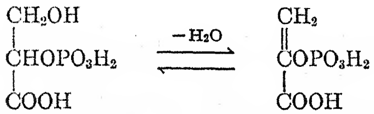


Creatine phosphate (N—P)



Phospho*enol*pyruvic acid (C—O—P)

As an interesting example of the formation of energy-rich bonds in alcoholic fermentation, the conversion of 2-phosphoglyceric acid to phospho*enol*pyruvic acid by removal of water may be cited. In this reaction, there is a transformation of an energy-poor ester linkage into an energy-rich C—O—P linkage:



2-Phosphoglyceric acid Phospho*enol*pyruvic acid

Although the energies represented by the two compounds are equal, the distribution within the compounds is such that energy is concentrated at the phosphate bond of the *enol*pyruvic acid. Lipmann (1941) in an excellent discussion points out that the dehydration of *free* glyceric acid to pyruvic acid must be exergonic—that is, it must occur with a loss of energy dissipated to the environment, whereas the dehydration of *phospho*glyceric acid to *enol*phosphopyruvic acid involves no energy loss because, through attachment of the phosphate group, the energy that would otherwise be dissipated is retained in the *enol* phosphate bond.

The mechanism of carbohydrate breakdown by the fungi is not as yet clear. Nord and Engel (1938) and Nord (1939, 1940) found two types of fermentation in *Fusarium*—one not involving phosphorylation during the early growth but followed by a phosphorylative mechanism.

The Phosphate Cycle. It is apparent from the scheme of alcoholic fermentation (Fig. 2, page 299) that phosphate undergoes a constant cyclic transfer. Inorganic phosphate is first introduced into the carbohydrate molecule in ester linkage. Primary phosphorolysis of glycogen or starch

occurs (Cori, 1939; Ostern *et al.*, 1936; Parnas, 1937); the reaction is reversible but the linkage is not energy-rich:

$$\text{glycogen} + \text{phosphate} \rightleftharpoons \text{glucose-1-phosphate}$$

The reaction is catalyzed by an enzyme containing adenylic acid as a prosthetic group (Cori, 1939). It appears that the adenylic acid acts as a phosphorylating catalyst without itself becoming intermediately phosphorylated.

Phosphate can also enter into molecules containing carboxyl groups (Lipmann, 1939):

$$\underset{\text{Pyruvic acid}}{CH_3{-}CO{-}COOH} + H_3PO_4 \xrightarrow{-2\,H} \underset{\text{Acetyl phosphate}}{CH_3C{\genfrac{}{}{0pt}{}{\diagup O}{\diagdown O{-}H_2PO_3}}} + CO_2$$

$$\underset{\text{Phosphoglyceraldehyde}}{CH_2O(H_2PO_3){-}CHOH{-}CHO} + H_3PO_4 \xrightarrow{-2\,H} \underset{\text{Diphosphoglyceric acid}}{CH_2O(H_2PO_3){-}CHOH{-}C{\genfrac{}{}{0pt}{}{\diagup O}{\diagdown OH_2PO_3}}}$$

In both reactions oxidation leads to the formation of an energy-rich phosphate bond. Considerable evidence has existed that the oxidation of pyruvic acid yields an active acetic acid because of the acetylation and condensation reactions which follow (Lipmann, 1940, 1941). The oxidation apparently passes through acetate. Lipmann has recently identified the active acetate as acetyl phosphate (Lipmann, 1937, 1939). Pyruvate was found to be oxidized by *Lactobacillus delbrueckii* to acetic acid and only in the presence of orthophosphate which forms an energy-rich bond, *i.e.*, adenosine triphosphate. Ochoa, Peters, and Stocken (1939) concluded that acetyl phosphate was not an intermediate in brain metabolism since the synthetic product was not oxidized. This failure was probably to be ascribed to failure to reproduce natural conditions.

$$2\ \text{acetyl phosphate} + \text{adenylic acid} \longrightarrow 2\ \text{acetic acid} + \text{adenosine triphosphate}$$

Only recently has the role of phosphate in the oxidation of carbonyl groups become apparent. It has been generally accepted that the oxidation of such groups required hydration, *e.g.*,

$$-C{\genfrac{}{}{0pt}{}{\diagup H}{\diagdown O}} + H_2O \longrightarrow -C{\genfrac{}{}{0pt}{}{\diagup H}{\genfrac{}{}{0pt}{}{-OH}{\diagdown OH}}} \longrightarrow -COOH + 2\,H$$

It is now apparent that phosphate and not water is taken up (Kalckar, 1941):

$$\underset{\text{Carbonyl phosphate}}{-C{\genfrac{}{}{0pt}{}{\diagup H}{\genfrac{}{}{0pt}{}{-O{-}P{\genfrac{}{}{0pt}{}{\diagup O}{\genfrac{}{}{0pt}{}{-OH}{\diagdown OH}}}}{\diagdown OH}}}} \rightleftharpoons \underset{\text{Carboxyl phosphate}}{-\underset{\Vert}{\overset{}{C}}{-}O{-}P{\genfrac{}{}{0pt}{}{\diagup O}{\genfrac{}{}{0pt}{}{-OH}{\diagdown OH}}}} + 2\,H$$

Hydration and oxidation involves a large decrease in free energy because of the formation of a resonating carboxyl:

whereas the decrease in free energy of the oxidation of carboxyl phosphate is small (since reaction is reversible). Thus the energy of the carboxyl group is retained to be transferred to structures where it can be utilized in performing work. The phosphate of carboxyl phosphate is transferred directly by a specific enzyme to form adenosine polyphosphate.

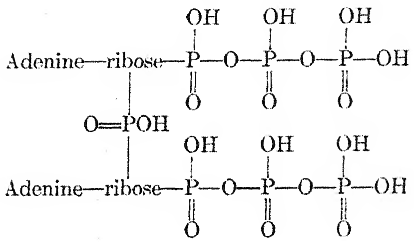
Fermentation represents a series of anaerobic reactions in which the energy made available by oxidation of carbohydrate is converted into phosphate bond energy. Two energy-rich phosphate bonds are formed for each triose oxidized, diphosphoglycerate containing one energy-rich bond and phospho*enol*pyruvate another. The *enol* phosphate arises from primary introduced ester phosphate and the acyl phosphate from the dehydrogenation of the addition product of inorganic phosphate and carboxyl compound. Since yeast does not attack starch, which must be hydrolyzed, glucose serves as the initial substrate of the yeast. Glucose is phosphorylated to form glucose-6-phosphate by the energy-rich adenosine triphosphate; thus, one energy-rich phosphate bond is used to form an ester linkage. This use of one energy-rich bond of adenosine triphosphate to form an ester linkage decreases the efficiency of the process since starch or glycogen already possesses the glucosidic linkage of equivalent value.

High-energy organic phosphate compounds are thus formed through the action of biological oxidations which maintain the energy supply at a high and constant level in actively metabolizing cells. This energy can be used to bring about the transfers of phosphate which must occur within the cell and are brought about by enzymes functioning much as do the hydrogen-transporting enzymes. Adenylic acid functions as a coenzyme to transfer phosphate either between high-energy compounds or to the lower ester level which is ultimately reached. Phosphate is generally split off by a phosphatase to the inorganic form, the lowest energy level. The reaction from the high energy level of approximately 10,000 cal to the ester level (*e.g.*, adenosine triphosphate to glucose-6-phosphate) involves a loss of 6000 cal. Transphosphorylation between organic molecules never passes through an inorganic phosphate stage since this would involve a loss in energy potential which would require a coupled oxidation to restore (Meyerhof *et al.*, 1938; Korzybski and Parnas, 1939).

Adenosine diphosphate and adenosine triphosphate are two important catalysts of alcoholic fermentation with a P—O—P linkage that function by virtue of their energy-rich phosphate linkages; there is one

such group in the diphosphate and there are two in the triphosphate. In both compounds, the remaining phosphate group is not energy-rich and does not enter into the reactions of fermentation. It is linked to ribose in an ordinary energy-poor ester linkage.

Diadenosine polyphosphate compounds have been isolated by Deuticke (1932), by Ostern (1934), and by Warburg and Christian (1936). Kiessling and Meyerhof (1938) isolated a diadenosine tetraphosphate with two easily hydrolyzable phosphate groups, possessing probably a phosphate bridge connecting the two ribose groups:



The adenosine polyphosphates are formed from adenosine monophosphate (adenylic acid) and phosphate in a reaction coupled with the oxidation of glyceraldehyde phosphate by coenzyme I (cozymase) to a mixture of the 3- and 2-phosphoglyceric acids (Meyerhof, Schulz, and Schuster, 1937; Needham and Pillai, 1938). Inhibitors of the oxidation prevent the formation of adenosine triphosphate to a corresponding degree. Warburg and Christian (1941) clarified the relationship by their discovery that 3-phosphoglyceraldehyde takes up phosphate to form 1,3-diphosphoglyceraldehyde, which is simultaneously oxidized to 1,3-diphosphoglyceric acid.

Warburg and Christian (1939) and Negelein and Brömel (1939a) studied the reaction series:

(a) coenzyme I + 3-phosphoglyceraldehyde + H_3PO_4 $\rightleftharpoons$
reduced coenzyme I + 1,3-diphosphoglyceric acid

(b) 1,3-diphosphoglyceric acid + adenosine diphosphate $\rightleftharpoons$
3-phosphoglyceric acid + adenosine triphosphate

They showed that, if the phosphate is not removed by an acceptor (adenosine diphosphate), the reaction will cease to flow to the right; an abnormal condition then occurs. Lipmann (1941) refers to a "pathological" reaction and cites the work of Shorr *et al.* (1940) as suggestive of such a deficiency of phosphate turnover in diabetic tissue.

Recently Meyerhof and Junowicz-Kocholaty (1943) questioned the occurrence of 1,3-diphosphoglyceraldehyde as an intermediate. They explain the reaction by proposing that the 3-phosphoglyceraldehyde and the

inorganic phosphate are simultaneously absorbed on the triose phosphate dehydrogenase. One hydrogen atom from the aldehyde and one from the phosphate are removed to effect the formation of the 1,3-diphosphoglyceric acid.

The classical equation of Harden (1932) for the fermentation of glucose by brewers' yeast juice is another such example:

$$2 \text{ hexose} + 2\ H_3PO_4 \longrightarrow \text{hexose diphosphate} + 2 \text{ ethanol} + 2 \text{ carbon dioxide}$$

Hexose diphosphate accumulates in the juice and thus prevents the regeneration of the inorganic phosphate that is needed in the cycle. This condition leads to cessation of fermentation until inorganic phosphate is added. That the total fermentation of yeast juice is stimulated by adding phosphate was observed by Wroblewski (1901), and by Buchner, Buchner, and Hahn (1903), who ascribed the increase to hydrogen-ion effects.

It was Harden, however, who first appreciated the fundamental role played by phosphate in the mechanism of alcoholic fermentation. When soluble phosphate was added to a yeast-juice fermentation of glucose (or levulose or mannose), the rate of fermentation rose rapidly and continued at high value for a certain period, determined by the presence of available phosphate, before falling. Harden and Young (1906) found that the "extra" carbon dioxide liberated as a result of adding phosphate was equivalent to the phosphate added. This fact indicates that a definite chemical reaction involving the phosphate and sugar occurs; Harden and Young (1905) showed that the phosphate had been esterified with sugar.

It is the "excessive" phosphorylation to form hexose diphosphate that brings the fermentation rate of juice to a very low value. If the phosphate enters and leaves the cycle at the same rate, it will serve as a true catalyst, although, in the early stages of ester formation carried out with bakers' yeast, some accumulation of phosphate ester does occur (Lipmann, 1938).

In preparing the yeast juice used by Harden and Young, the normal balance of reactions occurring in the intact cell is seriously disturbed; probably the phosphatases liberating inorganic phosphate are impaired; thus enough adenosine polyphosphate is formed to phosphorylate glucose, to form ultimately an excess of fructose-1,6-phosphate which accumulates. When sufficient adenosine polyphosphatases are formed, the amounts of adenosine di- and triphosphates are adequately controlled by the dephosphorylating action of the enzymes.

BIBLIOGRAPHY

ADAIR, G. S. (1928). A theory of partial osmotic pressures and membrane equilibria, with special reference to the application of Dalton's law to haemoglobin solutions in the presence of salts. *Proc. Roy. Soc. London* A120:573–603.

BEAR, R. S., and CORI, C. F. (1941). X-ray diffraction studies of synthetic polysaccharides. *J. Biol. Chem.* 140:111–118.

BIRCH, T. W., and MAPSON, L. W. (1936). Role of adenylic acid in vitamin B_1 deficiency. *Nature* 138:27–28.

BOYLAND, E., and BOYLAND, M. E. (1938). Studies in tissue metabolism. XI. The action of tumor slices and extracts on different carbohydrates. *Biochem. J.* 32:321–331.

BUCHNER, E., BUCHNER, H., and HAHN, M. (1903). *Die Zymasegärung. Untersuchungen über den Inhalt der Hefezellen und die biologische Seite des Gärungsproblems.* Oldenbourgh, Munich.

COLOWICK, S. P., and KALCKAR, H. M. (1941). An activator of the hexokinase system. *J. Biol. Chem.* 137:789–790.

——, WELCH, M. S., and CORI, C. F. (1940). Phosphorylation of glucose in kidney extract. *J. Biol. Chem.* 133:359–373.

CORI, C. F. (1939). Enzymatic breakdown and synthesis of carbohydrate. In *Cold Spring Harbor Symposia Quant. Biol.* 7:260–268.

— (1942). Phosphorylation of carbohydrates. In *A Symposium on Respiratory Enzymes*, pp. 175–189. Univ. Wisconsin Press, Madison.

——, COLOWICK, S. P., and CORI, G. T. (1937). The isolation and synthesis of glucose-1-phosphoric acid. *J. Biol. Chem.* 121:465–477.

—— and CORI, G. T. (1936). Mechanism of formation of hexosemonophosphate in muscle and isolation of a new phosphate ester. *Proc. Soc. Exptl. Biol. Med.* 34:702–705.

CORI, G. T., COLOWICK, S. P., and CORI, C. F. (1938). The enzymatic conversion of glucose-1-phosphoric ester to 6-ester in tissue extract. *J. Biol. Chem.* 124:543–555.

——, ——, and —— (1938a). The formation of glucose-1-phosphoric acid in extracts of mammalian tissues and of yeast. *J. Biol. Chem.* 123:375–380.

—— and CORI, C. F. (1940). The kinetics of the enzymatic synthesis of glycogen from glucose-1-phosphate. *J. Biol. Chem.* 135:733–756.

——, ——, and SCHMIDT, G. (1939). The role of glucose-1-phosphate in the formation of blood sugar and synthesis of glycogen in the liver. *J. Biol. Chem.* 129:629–639.

DEUTICKE, H. J. (1932). Über den Einfluss von Adenosin und Adenosinphosphorsäuren auf den isolierten Meerschweinchenuterus. *Arch. ges. Physiol. Pflügers* 230:537–555.

EMBDEN, G., DEUTICKE, H. J., and KRAFT, G. (1933). Über die intermediären Vorgänge bei der Glykolyse in der Muskulatur. *Klin. Wochschr.* 12:213–215.

GREEN, D. E., HERBERT, D., and SUBRAHMANYAN, V. (1940). On the isolation and properties of carboxylase. *J. Biol. Chem.* 135:795–796.

——, ——, and —— (1941). Carboxylase. *J. Biol. Chem.* 138:327–339.

—— and STUMPF, P. K. (1941). Starch phosphorylase. *J. Biol. Chem.* 140:47–48 (Proc.).

HANES, C. S. (1940). The breakdown and synthesis of starch by an enzyme system from pea seeds. *Proc. Roy. Soc. London* B128:421–450.

HARDEN, A. (1932). *Alcoholic Fermentation.* 4th ed. Longmans, Green, London.

—— and YOUNG, W. J. (1905). The influence of phosphates on the fermentation of glucose by yeast juice. Preliminary communication. *Proc. Chem. Soc.* 21: 189–191.

—— and —— (1906). The alcoholic ferment of yeast-juice. *Proc. Roy. Soc. London* B77:405–420.

HARDEN, A., and YOUNG, W. J. (1908). The alcoholic ferment of yeast-juice, Part III. The function of phosphates in the fermentation of glucose by yeast-juice. *Proc. Roy. Soc. London* **B80**:299–311.

HERBERT, D., GORDON, H., SUBRAHMANYAN, V., and GREEN, D. E. (1940). Zymohexase. *Biochem. J.* **34**:1108–1122.

KALCKAR, H. M. (1941). The nature of energetic coupling in biological syntheses. *Chem. Revs.* **28**:71–178.

—— (1942). The enzymatic action of myokinase. *J. Biol. Chem.* **143**:299–300.

KIESSLING, W. (1939). Über den das Glykogen phosphorylierenden Fermentproteinkomplex und eine enzymatische, reversible Glykogensynthese. *Biochem. Z.* **302**:50–72.

—— and MEYERHOF, O. (1938). Über ein Adenindinucleotid der Hefe: Di-(Adenosin-5′-phosphorsäure). *Biochem. Z.* **296**:410–425.

—— and SCHUSTER, P. (1938). Sterische Zugehörigkeit der biologische Glycerinsäure-α-phosphorsäure und Glycerinsäure-3-phosphorsäure. *Ber.* **71**:123–128.

KLUYVER, A. J., and STRUYK, A. P. (1928). The first phases of the chemistry of the dissimilation of the hexoses. (2nd part.) *Proc. Koninkl. Akad. Wetenschappen Amsterdam* **31**:882–893.

KORZYBSKI, T., and PARNAS, J. K. (1939). Observations sur les échanges des atomes du phosphore renfermés dans l'acide adénosinetriphosphorique, dans l'animal vivant, à l'aide du phosphore marqué par du radiophosphore. *Bull. soc. chim. biol.* **21**:713–716.

LIPMANN, F. (1937). Die Dehydrierung der Brenztraubensäure. *Enzymologia* **4**:65–72.

—— (1938). Gärversuche mit Mazerationsextrakten aus Bäckerhefe. *Compt. rend. trav. lab. Carlsberg, sér. chim.* **22**:317–320.

—— (1939). An analysis of the pyruvic acid oxidation system. *Cold Spring Harbor Symposia Quant. Biol.* **7**:248–259.

—— (1940). A phosphorylated oxidation product of pyruvic acid. *J. Biol. Chem.* **134**:463–464.

—— (1941). Metabolic generation and utilization of phosphate bond energy. In *Advances in Enzymology*, Vol. I, pp. 99–162. Interscience, New York.

LOHMANN, K. (1933). Über Phosphorylierung und Dephosphorylierung. Bildung der natürlichen Hexosemonophosphorsäure aus ihren Komponenten. *Biochem. Z.* **262**:137–151.

—— and MEYERHOF, O. (1934). Über die enzymatische Umwandlung von Phosphoglycerinsäure in Brenztraubensäure und Phosphorsäure. *Biochem. Z.* **273**:60–72.

—— and SCHUSTER, P. (1937). Untersuchungen über die Cocarboxylase. *Biochem. Z.* **294**:188–214.

LUNDSGAARD, E. (1938). The biochemistry of muscle. *Ann. Rev. Biochem.* **7**:377–398.

McFARLANE, M. G. (1937). Phosphorylation on living yeast. *J. Soc. Chem. Ind.* **56**:935.

MELNICK, D., and FIELD, H., JR. (1939). Chemical determination of vitamin B_1. III. Quantitative enzymic conversion of cocarboxylase (thiamine pyrophosphate) to the free vitamin. *J. Biol. Chem.* **127**:531–540.

MEYER, K. H., and BERNFELD, P. (1942). Recherches sur l'amidon XXI. Sur les enzymes amylolytiques de la levure. *Helv. Chim. Acta* **25**:399–403.

MEYERHOF, O. (1935). Über die Wirkungsweise der Hexokinase. *Naturwissenschaften* **23**:850–851.

—— (1937). Über die Intermediärvorgänge der enzymatischen Kohlehydratspaltung. *Ergeb. Physiol. Pharmakol.* **39**:10–75.

—— (1941). Oxidoreductions in carbohydrate breakdown. *Biol. Symposia* **5**:141–156.

—— (1942). Intermediate carbohydrate metabolism. In *A Symposium on Respiratory Enzymes*, pp. 3–15. Univ. of Wisconsin Press, Madison.

MEYERHOF, O., and JUNOWICZ-KOCHOLATY, R. (1942). The two-fold activation of carbohydrate breakdown by arsenate and the dephosphorylation of phosphopyruvic acid. *J. Biol. Chem.* 145:443–456.

—— and —— (1943). The equilibria of isomerase and aldolase, and the problem of the phosphorylation of glyceraldehyde phosphate. *J. Biol. Chem.* 149:71–92.

—— and KIESSLING, W. (1933). Zwischenprodukte des Kohlenhydratumsatzes im Muskelextrakt. *Naturwissenschaften* 21:223–224.

—— and —— (1934). Über ein neues phosphoryliertes Intermediärprodukt der Kohlehydratspaltung und sein enzymatisches Gleichgewicht. *Naturwissenschaften* 22:838.

—— and LOHMANN, K. (1934). Über die enzymatische Gleichgewichtsreaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. *Biochem. Z.* 271:89–110.

—— and —— (1934a). Über die enzymatische Gleichgewichtsreaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. II. Die negative Wärmetönung der enzymatischen Spaltung. *Biochem. Z.* 273:73–79.

—— and —— (1934b). Über die enzymatische Gleichgewichtsreaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. III. Über Abfangen der Triosephosphorsäure mit Bisulfit und die Verbreitung des Ferments Zymohexase in den verschiedenen Zellarten. *Biochem. Z.* 273:413–418.

—— and —— (1935). Über die enzymatische Gleichgewichtsreaktion zwischen Hexosediphosphorsäure und Dioxyacetonphosphorsäure. IV. (Berichtigung zur II. Mitteilung.) *Biochem. Z.* 275: 430–432.

——, ——, and SCHUSTER, P. (1936). Über die Aldolase, ein kohlenstoffverknüpfendes Ferment. II. Aldolkondensation von Dioxyacetonphosphorsäure mit Glycerinaldehyd. *Biochem. Z.* 286:319–335.

—— and OHLMEYER, P. (1937). Über die Rolle der Co-Zymase bei der Milchsäurebildung im Muskelextrakt. *Biochem. Z.* 290:334–353.

——, ——, GENTNER, W., and MAIER-LEIBNITZ, H. (1938). Studium der Zwischenreaktionen der Glykolyse mit Hilfe von radioaktivem Phosphor. *Biochem. Z.* 298:396–411.

——, ——, and MÖHLE, W. (1938). Über die Koppelung zwischen Oxydoreduktion und Phosphatveresterung bei der anaeroben Kohlenhydratspaltung. *Biochem. Z.* 297:113–133.

—— and SCHULZ, W. (1935). Über die Energieverhältnisse bei der enzymatischen Milchsäurebildung und der Synthese der Phosphagene. *Biochem. Z.* 281:292–305.

——, ——, and SCHUSTER, P. (1937). Über die enzymatische Synthese der Kreatinphosphorsäure und die biologische "Reaktionsform" des Zuckers. *Biochem. Z.* 293:309–337.

NEEDHAM, D. M., and PILLAI, R. K. (1938). The coupling of oxidoreductions and dismutations with esterification of phosphate in muscle. *Biochem. J.* 31:1837–1851.

NEGELEIN, E., and BRÖMEL, H. (1939). R-Diphosphoglycerinsäure, ihre Isolierung und Eigenschaften. *Biochem. Z.* 303:132–144.

—— and —— (1939a). Isolierung eines reversibeln Zwischenprodukts der Gärung. *Biochem. Z.* 301:135–136.

NILSSON, R., and ALM, F. (1936). Zur Kenntnis der alkoholischen Gärung in dem intakten Fermentsystem der Hefezelle und in desorganisierten Zymasesystemen. *Biochem. Z.* 286:254–278, 373–391.

—— and —— (1940). Zur Kenntnis der alkoholischen Gärung in dem intakten Fermentsystem der Hefezelle und in desorganisierten Zymasesystemen. III. Die Bedeutung der Zellstruktur für den harmonischen Gärverlauf. *Biochem. Z.* 304:285–317.

NORD, F. F. (1939). Enzymatische Umsetzungen durch Fusarien: Beitrag zum Mechanismus der alkoholischen Gärung. *Ergeb. Enzymforsch.* 8:149–184.

—— (1940). Facts and interpretations in the mechanism of alcoholic fermentation. *Chem. Revs.* 26:423–472.

NORD, F. F., and ENGEL, W. (1938). Beobachtungen bei der Vergärung von Biosen durch *Fusarium lini* Bolley. IV. Mitteilung zur Biochemie der Fusarien. 17. Mitteilung zum Mechanismus der Enzymwirkung. *Biochem. Z.* 296:153–170.

OCHOA, S. (1941). Glycolysis and phosphorylation in brain extracts. *J. Biol. Chem.* 141:245–251.

—— and OCHOA, C. G. (1937). Cozymase in invertebrate muscle. *Nature* 140:1097.

—— and PETERS, R. A. (1938). Vitamin B_1 and cocarboxylase in animal tissues. *Biochem. J.* 32:1501–1515.

——, ——, and STOCKEN, L. A. (1939). Acetylphosphate and pyruvate oxidation. *Nature* 144:750–751.

OSTERN, P. (1934). Über Di-Adenosin-pentaphosphorsäure (Herznucleotid). *Biochem. Z.* 270:1–5.

——, GUTHKE, J. A., and TERSZAKOWEC, J. (1936). Über die Bildung des Hexosemonophosphorsäure-esters und dessen Umwandlung in Fructose-diphosphorsäure-ester in Muskel. *Z. physiol. Chem.* 243:9–37.

——, ——, and —— (1936a). Les transformations initiales de la glycogénolyse. La fonction de l'ester hexosemonophosphorique. *Compt. rend. soc. biol.* 121: 282–285.

PARNAS, J. K. (1936). L'enchainement des processus enzymatiques dans le tissu musculaire. *Bull. soc. chim. biol.* 18:53–85.

—— (1937). Der Mechanismus der Glykogenolyse im Muskel. *Ergeb. Enzymforsch.* 6:57–110.

—— and BARANOWSKI, T. (1935). Sur les phosphorylations initiales du glycogéne. *Compt. rend. soc. biol.* 120:307–310.

PILLAI, R. K. (1938). Dephosphorylation in muscle extracts. *Biochem. J.* 32: 1087–1099.

POTTER, V. R. (1944). Biological energy transformations and the cancer problem. In *Advances in Enzymology*, Vol. IV, pp. 201–250. Interscience, New York.

ROBISON, R. (1922). A new phosphoric ester produced by the action of yeast juice on hexoses. *Biochem. J.* 16:809–824.

SCHLENK, F. (1942). Nicotinamide nucleotide enzymes. In *A Symposium on Respiratory Enzymes*, pp. 104–133. Univ. Wisconsin Press, Madison.

—— and EULER, H. v. (1936). Cozymase. *Naturwissenschaften* 24:794–795.

SCHULTZ, A., ATKIN, L., and FREY, C. N. (1937). A fermentation test for vitamin B_1. *J. Am. Chem. Soc.* 59:948–949. A fermentation test for vitamin B_1, II. *Ibid.*, 2457–2460.

——, ——, and —— (1938). Influence of nicotinic acid on the fermentation method for vitamin B_1 determination. *J. Am. Chem. Soc.* 60:1514–1515.

SCHWANN, T. (1837). Vorläufige Mittheilung, betreffend Versuche über die Weingährung und Fäulnis. *Ann. Physik Chem.* 41:184–193.

—— (1839). *Mikroskopische Untersuchungen über die Übereinstimmung in der Struktur und dem Wachsthum der Thiere und Pflanzen.* Englemann, Leipzig.

SHORR, E., BARKER, S. B., COHEN, E., and MALAM, M. (1940). Further studies on the restoration of carbohydrate oxidation to diabetic tissue in vitro in the absence of insulin. *Am. J. Physiol.* 129:463–464.

SUMNER, J. B., and SOMERS, G. F. (1943). *Chemistry and Methods of Enzymes.* Academic Press, New York.

SUTHERLAND, E. W., COLOWICK, S. P., and CORI, C. F. (1941). The enzymic conversion of glucose-6-phosphate to glycogen. *J. Biol. Chem.* 140:309–310.

TAUBER, H. (1938). The interaction of vitamin B_1 in enzymic reactions. *J. Biol. Chem.* 123:499–506.

UTTER, M. F., and WERKMAN, C. H. (1942). Effect of metal ions on the reactions of phosphopyruvate by *Escherichia coli.* *J. Biol. Chem.* 146:289–300.

VALLERY-RADOT, R. (1937). *The Life of Pasteur.* Translated by Mrs. R. L. Devonshire. Garden City, New York.

WARBURG, O., and CHRISTIAN, W. (1936). Pyridin, der wasserstoffübertragende Bestandteil von Gärungsfermenten. (Pyridin-Nucleotide.) *Biochem. Z.* **287**:291–328.

—— and —— (1939). Proteinteil des kohlenhydrat-oxydierenden Ferments der Gärung. *Biochem. Z.* **301**:221–222.

—— and —— (1939a). Isolierung und Kristallization des Proteins des oxydierenden Gärungsferments. *Biochem. Z.* **303**:40–68.

—— and —— (1941). Isolation and crystallization of enolase. *Naturwissenschaften* **29**:589–590.

——, KUBOWITZ, F., and CHRISTIAN, W. (1930). Über die katalytische Wirkung von Methylenblau in lebenden Zellen. *Biochem. Z.* **227**:245–271.

WEIL-MALHERBE, H. (1939). The enzymic phosphorylation of vitamin B_1. *Biochem. J.* **33**:1997–2007.

WERKMAN, C. H. (1939). Bacteriological dissimilation of carbohydrates. *Bact. Revs.* **3**:187–225.

—— and WOOD, H. G. (1942). On the metabolism of bacteria. *Botan. Rev.* **8**:1–68.

WIGGERT, W. P., and WERKMAN, C. H. (1930). Phosphorylation by the living bacterial cell. *Biochem. J.* **32**:101–107.

WROBLEWSKI, A. (1901). Ueber den Buchner'schen Hefepressaft. *J. prakt. Chem.* **64**:1–70.

YOUNG, W. J. (1909). The hexosephosphate formed by yeast-juice from hexose and phosphate. *Proc. Roy. Soc. London* **B81**:528–545.

CHAPTER XI

YEAST FERMENTATION

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As the mechanism of panary fermentation yields to experimental investigation it becomes increasingly apparent that the process is a dynamic one operating in a shifting environment. Flour alone is a varied and complex mixture, but the formation of a dough initiates a series of chemical and physical changes which do not cease even when the dough has been baked into bread. The factors directly and indirectly connected with the leavening action (carbon dioxide production) of the living yeast account for a large part of the complexity. The yeast reacts at different rates with at least three of the four different sugars. And the concentrations of these are continually changing; some because the supply is exhausted, and one because it is constantly being produced by other dough ingredients. Furthermore, the yeast absorbs or reacts with several salts, vitamins, and other organic compounds. The concentration of these factors also changes during the life of the dough as a consequence of the activity of proteases, phytase, phosphatase, and perhaps other enzymes. Furthermore, other aspects of the environment change, from aerobic to anaerobic and from *p*H 6 to *p*H 5. The whole dynamic system is necessarily studied with variable flour, different yeasts, and with varied additions to the formula. It is unlikely that in our time the last word will be written about fermentation.

Flour is not sterile; consequently bacteria and molds are sometimes responsible for changes in the dough. Recent work (Freilich and Frey, 1939, 1941, 1943) also indicates that the activity of dough enzymes can be inhibited or accelerated by changing the oxygen content of the atmosphere in which doughs are mixed. The effect of mixing on bromate action, and the action of yeast in affecting bromate decomposition by means of acidity changes in the dough, indicate the wide field of research now open to us.

One of the more important features of the present discussion is the

role of thiamine (vitamin B_1) in dough fermentation. Previous studies had shown that fermentation of a sugar solution was but a weak counterpart of dough fermentation, and consequently there was a justifiable lack of confidence in interpretation of dough fermentation and bread quality based on these studies. Although it cannot be said that all doubt has been eliminated, the attainment of dough fermentation rates in solutions of defined composition is considered to be a significant forward step. The principal discovery has been that thiamine was the factor missing from previous studies.

That dough fermentation is an enzymatic process is quite obvious. It is equally obvious that the yeast cell constitutes an enzyme system which is organized in a manner peculiar to living tissues. Few, if any, of the enzymes of yeast are independent of the other enzymes of the system. For the most part, only the end-result of a series of reactions is observed, and it is upon the end-result that a practical understanding of dough fermentation must be based. The present chapter deals only with the reactions of living yeast, there being no evidence that dough fermentation is brought about by cell-free fermentation, although such a possibility is not excluded.

The literature of alcoholic fermentation is very extensive and no attempt has been made to cover it all, except in a limited field; instead, reliance is placed upon selected references, and those interested in following a particular phase to its origin may use these references as starting points.

THE EQUATION OF FERMENTATION

The fundamental equation of alcoholic fermentation, sometimes called the Gay-Lussac equation, is:

$$\underset{\text{Zymohexose}}{C_6H_{12}O_6} \longrightarrow \underset{\text{Alcohol}}{2\,C_2H_5OH} + \underset{\text{Carbon dioxide}}{2\,CO_2}$$

This reaction is probably never realized exactly because, as Pasteur has shown, succinic acid, glycerol, and probably other substances are normal by-products of fermentation. In addition, a certain proportion of the sugar may be used by the yeast for nutritive purposes. Since the leavening action of fermentation is due to the gas evolved, present attention is directed to the yield of carbon dioxide per gram of sugar removed from solution. According to the above equation, one mole (180.2 g) of glucose yields 2 moles (88 g) of carbon dioxide which has a volume of 44.8 liters at N.T.P. This corresponds to 249 ml of gas per g of sugar. When corrected for water vapor and expansion to 25°C, this volume is 281 ml. Van Niel and Anderson (1941) have obtained very nearly the theoretical maximum volume of gas using the method and apparatus of Kluyver (1914). In Kluyver's method, the yeast remains in the sugar solution for 16 to 24 hours, a condi-

tion which does not occur in bread manufacture under ordinary circumstances. Van Niel and Anderson have shown that, under conditions of cell density and sugar concentration wherein the time taken for the complete disappearance of sugar from the solution is only 1 hour, only about 70 per cent of the theoretical carbon dioxide is evolved. They have further shown that, under these conditions, there is an increase in dry weight of the yeast which corresponds to a significant part of the fermentation deficit. Willstätter and Rohdewald (1937) claim that the fermentation of hexose sugars proceeds via the formation of polysaccharides within the yeast cell, which might be viewed as consistent with the results mentioned above. After the sugar has disappeared from the solution, there is a slow but measurable evolution of carbon dioxide from the yeast suspension (Van Niel and Anderson, 1941), which in all probability accounts for the higher gas yields obtained by Kluyver's method.

It may therefore be deduced that under conditions of fairly rapid fermentation the gas yield will be considerably less than theoretical. Schultz and Kirby (1933), with their biological method for the determination of sugars, obtain 200 ml of gas per g of glucose or maltose and 210 ml per g of sucrose. As mentioned by Schultz, Atkin, and Frey (1937), the addition of thiamine to the fermentation medium increases the yield of gas. Schultz, Fisher, Atkin, and Frey (1943) employ "Hi-B_1" bakers' yeast (rich in thiamine) or regular bakers' yeast plus an excess of thiamine, and obtain about 215 ml per g of glucose or maltose and 225 ml for sucrose. These average figures were not included in the publication referred to because the gas equivalence, measured in each determination, naturally varies somewhat with atmospheric conditions such as temperature and pressure. The efficiency under average conditions is about 75 per cent.

It is not feasible to assess the gas yield directly in a dough because the formation of new sugar hardly ceases at any time; furthermore, the total sugar formed is difficult to estimate at any instant inasmuch as yeast-free doughs form less sugar than fermenting doughs (Landis, 1934). The figures of Schultz *et al.* are useful, however, in estimating from gas volumes the amount of sugar fermented in fermenting doughs or suspensions. When conditions permit the methods are best calibrated with known increments of pure sugar.

INFLUENCE OF TEMPERATURE AND HYDROGEN-ION CONCENTRATION

The Temperature Coefficient. Temperature has a large effect upon the velocity of fermentation. Slator (1906) found the same coefficients for a brewery yeast, a distillery yeast, and a wine yeast. A summary of his

results showing the temperature coefficient of fermentation (V_{t+10}/V_t), at temperatures from 10 to 35°C, follows:

Temperature	10°	15°	20°	25°	30°	35°
Temperature coefficient	5.6	3.8	2.8	2.25	1.9	1.6

The temperature coefficients of fructose and sucrose fermentation are similar to those of glucose over the same range, but maltose has a significantly higher coefficient below 25°C (Slator, 1908). In the range 25 to 35° all of the common sugars show similar coefficients.

Guillemet and Schell (1934) used bakers' yeast in sugar solutions and in wheat-flour pastes, and observed that fermentation is three times as rapid at 30°C as at 20°C. Thus they obtain a coefficient of 3 as compared with Slator's 2.8. In unpublished experiments at the Fleischmann laboratories, a glucose fermentation rate twice as great at 35°C as at 25°C has been observed, which is in fair agreement with Slator's value of 2.25.

Beyond the obvious influence on fermentation time and proof time, changing the temperature of the dough does not appear to have any serious effect upon the bread (Fisher and Halton, 1937). Doughs maintained at 65°, 75°, 85°, and 95°F were proofed to uniform volume and then baked. Except for the 65° loaf, all were judged to be alike in quality. The judging of bread quality is a complex problem, and Ekstedt (1938) has reported a considerable difference of opinion among bakery engineers concerning the effect of temperature on dough handling, and also the effect on the finished product. The large temperature coefficient of fermentation makes it essential that laboratory tests be conducted with careful temperature control. In shop practice it is evident that temperature fluctuations will cause corresponding changes in dough schedules.

Influence of Hydrogen-Ion Concentration. In general, yeast exhibits maximum activity, both in fermentation and respiration, on the acid side of neutrality. Trautwein and Wassermann (1931) studied the behavior of both top and bottom brewers' yeast in potassium phosphate buffer solutions. They observed a fairly broad optimum range for fermentation (between pH 4.0 and 6.0), which fell off rather rapidly on either side and showed a peak at pH 4.7. Leibowitz and Hestrin (1942), on the other hand, found no change in the rate of glucose fermentation by bakers' yeast between pH 3.0 and 7.0. These workers found that pH 4.0 to 6.0 is the optimum range for maltose fermentation with the same yeast. The question of the optimum acidity for breadmaking, or of whether there is any single optimum, has been a matter of dispute since Jessen-Hansen (1911) did his pioneer work. Present concern, however, is directed only to the influence of hydrogen-ion concentration upon yeast activity and vice versa. Fisher and Halton (1929, 1929a) added acids and bases to doughs and studied, among

other things, the influence of acidity on gas production. They conclude that an increase of acidity to well below *p*H 5 has no significant effect on gas production up to the fourth hour of fermentation. After this time the effect was found to be variable, but in general there was a slight increase in gas production on the acid side of *p*H 5.0.

Changes in Acidity during Fermentation. Bailey and Sherwood (1923) were among the first in America to study the march of hydrogen-ion concentration in bread doughs. They found that the acidity increases at a fairly uniform rate in bread doughs fermented under fixed conditions. In a 4-hour period, the change in hydrogen-ion concentration of laboratory straight doughs averages about 0.41 *p*H unit, and in commercial straight doughs about 0.47 *p*H unit. Increasing the temperature of the dough accelerates the rate of increase in acidity. In 1000-pound straight doughs the rate of change in acidity is somewhat more rapid than in 1-pound laboratory doughs. When acid or acid salts are added to the dough, the hydrogen-ion concentration is lowered and remains so throughout the fermentation period. Arkady does not appreciably affect the initial hydrogen-ion concentration of the dough, but causes it to increase at a more rapid rate than in the control dough. The grade of flour bears an important relation to rate of change of acidity in doughs, the high-grade or patent flour doughs changing more rapidly than low-grade or clear-flour doughs. Depending on the flour and other ingredients, the initial acidity of the dough may vary from *p*H 5.36 to 6.2.

Fisher and Halton (1929, 1929a) and Landis (1934) observed similar trends. St. John and Bailey (1929) found that skim milk solids decreased the initial hydrogen-ion concentration from 0.35 to 0.5 *p*H unit. Blish and Hughes (1932) made a comprehensive study of the changes produced in the dough in the course of fermentation using a single standard commercial "Bakers' Patent" flour throughout. Doughs containing 3 per cent of yeast and 0, 2.5, and 10 per cent of sugar were studied for 24 hours. Progressive changes in acidity followed essentially the same course regardless of the initial sugar concentration or the duration of the active period of fermentation, that is, downward to approximately *p*H 5.0 in 7 hours and then without alteration for the next 17 hours. These results do not agree with those of Johnson and Bailey (1924), who found that the acidity of cracker doughs steadily increased for 24 hours, ultimately reaching a value slightly below *p*H 4.0. Neither do these values conform to the findings of Johnson (1925) with flour–water suspensions containing yeast, in which *p*H 3.7 was reached in 24 hours. It is possible that the discrepancy is due to the action of nonyeast microorganisms which develop to some extent in fermentations of long duration.

The nature of the acidity produced during fermentation deserves

comment. Johnson (1925) found that about 75 per cent of the acidity formed in bread dough is lactic acid and that acetic acid constitutes the remaining 25 per cent. Blish and Hughes (1932) also found that the greater portion of the organic acids produced was nonvolatile (lactic acid).

INFLUENCE OF SUGARS

Concentration. It is generally stated that the concentration of sugar is without significant effect upon the rate of alcoholic fermentation in concentrations ranging from 0.5 to 10 per cent (Slator, 1906). But Hopkins and Roberts (1935) have shown that this is at best an approximation and depends to a large extent upon the choice of conditions. These workers employed brewery yeast and used simple sugar solutions. Their conclusions do not necessarily apply without modification to alcoholic fermentation in the dough where one finds fermentation-accelerating substances, buffers, etc. However, the fundamental behavior of the living yeast cell will, in all probability, have much in common under both conditions, and the observations of Hopkins and Roberts deserve close scrutiny. They conclude: " . . . if all the conditions which influence the rate of alcoholic fermentation of glucose by living yeast are carefully considered the Kinetics are precisely similar to those of an ordinary enzyme reaction." They showed that the fermentation of glucose conforms to the theory of Michaelis and Menten (1913) between 0.2 and 5 per cent glucose concentration and with 1 g of yeast per 100 ml. According to Haldane (1930), if an enzyme action follows the Michaelis and Menten equation, three cases can arise:

1. The substrate concentration remains so high that the enzyme is fully saturated and consequently the observed rate of reaction is nearly constant. If the enzyme concentration is sufficiently low, the observed rate of reaction will be nearly uniform over a fairly wide range of substrate concentrations. This is apparently the condition under which Slator (1906) made his observations.

2. The substrate concentration is so low that the amount of enzyme combined with substrate is proportional to substrate concentration, and the reaction behaves in a unimolecular manner. This condition was examined by Hopkins and Roberts and found to agree with theory.

3. The substrate concentration varies so that neither of the above conditions is fulfilled. Here the rate of fermentation may rise to a maximum, remain stationary for a period, and then drop as the substrate is exhausted. Hopkins and Roberts (1935) found that, at the relatively low yeast concentration of 1 g per 100 ml, the rate of fermentation varied comparatively little between 2 and 10 per cent of glucose, whereas with higher yeast concentration greater variations and more definite maxima were exhibited.

Flour contains upward of 1.0 per cent of sugar; sugar is usually added to the dough, and diastasis produces sugar as soon as the dough is mixed. Furthermore, the absorption is always less than 100 per cent. Consequently, it may be expected that the major part of dough fermentation occurs at moderate sugar concentrations, and under these conditions the fermentation rate will not be greatly influenced by variation in sugar concentration, unless unusually high yeast levels are employed. It should be borne in mind that the baker and cereal chemist express the sugar content of doughs as per cent of the flour in the formula. Thus, a dough containing 100 parts of flour, 70 parts of water, and 7 parts of sugar, will be described as containing 7 per cent of sugar. In terms of the water, the sugar concentration is 10 per cent. It is necessary for the baker to use this method of expression because the absorption or quantity of water necessary to form a satisfactory dough is variable.

Heald (1932) found no effect on fermentation rate in doughs when the sugar concentration was varied from 0 to 5 per cent, based on the flour. The data of Larmour and Brockington (1934) show that the initial rate of gas production in the dough is not affected in the range of 0 to 6 per cent at yeast levels of 1, 3, and 5 per cent.

The effect of very high sugar concentration on fermentation and the activity of microorganisms in general is well known, and is evidenced by the use of syrups, etc., in the preservation of various foods. The preservative action of high sugar concentration is considered to be due to the osmotic effect. As the concentration of sugar in the dough is increased much above 10 per cent, the rate of fermentation is progressively retarded. Other substances usually present in the dough also have an osmotic effect—salt, soluble solids of flour, and soluble milk solids. There is a scarcity of published work on the influence of high sugar concentration on the fermentation of doughs of various compositions. The inevitable retardation of fermentation rate in sweet doughs is commonly overcome by the use of significantly increased yeast concentration; up to 8 per cent of yeast based on the flour is frequently used.

Sources of Sugar in the Dough. The three sources of fermentable sugar in the dough are: first, sugar originally present in the flour; second, the maltose gradually produced by the action of the flour enzymes or other diastatic enzymes on starch; and third, the amount of sugar intentionally added as a dough ingredient. The nature and quantity of sugar in flour has been the subject of considerable work. Blish, Sandstedt, and Astleford (1932) reported that the quantity of reducing sugars present as such in normal, sound, bread flours is extremely small (probably 0.1 to 0.2 per cent) and nearly constant, but that the sucrose content is nearly ten times as great—it varies from 1.0 to 1.74 per cent. Earlier workers, Stone (1896)

and Teller (1912), obtained considerably lower figures. They used hot, 95 per cent alcohol to extract the sugars. Shutt (1908) used 75 per cent alcohol and obtained 0.05 to 0.26 per cent of reducing sugars calculated as maltose, and from 0.91 to 1.42 per cent of sucrose. The procedure developed by Blish *et al.* for the extraction of the sugars of flour calls for the extraction of 5 g of flour with 25 ml of a solution containing 6 ml of concentrated sulfuric acid per liter, followed by 25 ml of a 1 per cent sodium tungstate solution. All operations are conducted at ice-water temperatures. Reducing sugars are determined on an aliquot of the filtrate. For determination of sucrose, 50 ml of extract are treated with 5 ml of hydrochloric acid at room temperature overnight, and the reducing sugars are then determined after inversion. The work of Colin and Belval (1935) indicates that the percentage of sucrose in white flour may be considerably lower than 1 per cent. According to Colin and Belval, a typical white flour might contain 0.17 per cent of a hexose sugar, 0.22 per cent of sucrose, and 0.6 per cent of levosin.

Levosin, originally described by Tanret (1891), is described by Colin and Belval (1935a) as being a white amorphous substance, faintly sweet, and soluble in water and dilute alcohol. Its rotation is $[\alpha]_D = -36°$; and on acid hydrolysis it yields 1 mole of glucose and 9 moles of fructose. It is practically unfermentable in pure solution (Geoffroy, 1935), but Colin and Belval and Geoffroy agree that most of it disappears in the course of the fermentation of the dough. Guillemet (1935) found that about 30 per cent of the levosin was fermented in 24 hours when added to a yeast–water medium. The substance may be partially hydrolyzed by an enzyme in flour, since Colin and Belval found that, if flour is "sterilized" (*i.e.*, its enzymes destroyed), by boiling 95 per cent alcohol, the residue will yield only 0.15 per cent of alcohol upon fermentation. This observation indicates that there is a very small proportion of directly fermentable sugars originally present in flour. Practically, however, a sugar which is available for fermentation very early in the doughing process might have as much significance as though it were initially present.

One of the difficulties involved in determining the sugar originally present in flour is the rapid formation of maltose, and this is enhanced by the subsequent difficulty in distinguishing maltose from other sugars by chemical analysis. The experiment described in Table I* makes use of the technique described by Schultz and Kirby (1933) and Schultz, Fisher, Atkin, and Frey (1943). A sample of white flour was subjected to fermentation with yeast No. 2019 which ferments all sugars, except maltose, that are fermented by normal bakers' yeast. The fermentable sugars (not including maltose) are estimated by measuring the gas evolved. To compensate for dissolved gas, a measured amount of sucrose is fermented with and without

* Atkin, Schultz, and Frey, unpublished data.

the flour (tests 1 and 3) and the difference in evolved gas is assumed to result from the fermentable sugar in the flour. In the present case, the difference is 68 ml of gas. As mentioned previously, the gas equivalent to 1 g of sucrose under these conditions is 225 ml. Thus 30 g of the white flour contained 0.3 g, which is equivalent to about 1.0 per cent of fermentable sugars (not maltose). Whether this 1 per cent includes some levosin cannot be stated, but the results indicate that the quantity of readily available sugar, while not as high as some have found, is certainly not as low as others have stated.

TABLE I

DEMONSTRATION OF FERMENTABLE SUGARS IN FLOUR WITH "HI-B_1" YEAST NO. 2019 WHICH FERMENTS ALL SUGARS, EXCEPT MALTOSE, THAT ARE FERMENTED BY NORMAL BAKERS' YEAST

All substrates were mixed with 30 ml of water, and the gas was measured at atmospheric pressure.

Substrate	Gas evolved at 30°C after fermenting for:						
	15 min	30 min	45 min	60 min	75 min	90 min	105 min
	ml	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*
1. Cane sugar, 0.5 g Salt, 0.5 g	22	46	74	99	105	107	107
2. White flour, 30 g Salt, 0.5 g	8	25	38	45	50	55	58
3. White flour, 30 g Cane sugar, 0.5 g Salt, 0.5 g	4	42	82	125	155	169	175
4. Maltose, 0.5 g Salt, 0.5 g	3	2	1	0	−1	−1	−1

Perhaps the sugar most frequently added to the dough is cane sugar or sucrose. The amount varies quite widely. Frequently it is about 5 per cent based upon the flour; but the sugar content may be as high as 22 per cent in the so-called sweet doughs. Malt syrup is often used as a sugar source. Maltose is the principal sugar in malt syrup, although it contains some glucose, some dextrins, and cane sugar or invert sugar.

The diastatic enzymes of flour produce fermentable sugars when the flour is made into a dough. The normal diastatic activity of flour can be supplemented by the addition of enzymes during the milling process, or when the dough is mixed. Landis (1934) has shown that as much as 5 per cent of the flour weight may be converted to sugar during fermentation of the dough. The diastatic enzymes in the dough act upon only a small fraction of the available starch, which for some reason is attackable without preliminary heat treatment. The attackable starch in a dough may be increased by an addition of one of the various preparations of partially degraded starch or dextrins.

Influence of Glucose and Fructose. There are four fermentable sugars encountered by yeast in the dough: glucose, fructose, sucrose, and maltose. When milk solids are added to the dough, lactose is also present; but this sugar is not fermented directly by any variety of bakers' yeast. Glucose, fructose, and sucrose are fermented at very nearly the same rate according to Slator (1906, 1908). However, Hopkins and Roberts (1935a) have found that glucose is fermented at a slightly faster rate than fructose in the range of 2 to 8 per cent concentration, and much faster at lower concentrations. These workers found that the reaction kinetics for glucose in the range of 0.2 to 5 per cent, and for fructose between 0.4 and 6 per cent sugar concentration, follow the equation of Michaelis and Menten (1913). The Michaelis constant, K_m, is 0.01067 for glucose and 0.0225 for fructose. This signifies that the enzyme that controls the rate of fermentation under these conditions has a greater affinity for glucose than for fructose. These observations are in excellent agreement with the results of investigation of the relative rates of fermentation of glucose and fructose from mixtures of the two, which were made by Hopkins (1928), Sobotka and Reiner (1930), and Hopkins (1931), each of whom found that glucose is fermented at a faster rate.

The difference in fermentation rate is expressed by a constant, $K_{G/F}$, called the selectivity constant, which is defined by these authors as follows:

$$K_{G/F} = \frac{\log Y_0 - \log Y}{\log Z_0 - \log Z} \tag{1}$$

where Y_0 and Z_0 represent the initial concentrations of glucose and fructose respectively, and Y and Z represent the concentrations at any given time. Hopkins (1931) obtained values for $K_{G/F}$ of 1.79 to 3.8 for various yeasts with the exception of sauterne yeast, which showed a preference for fructose. Sobotka and Reiner (1930) obtained a constant of 2.46 for bakers' yeast. Typical of their results is an experiment in which the initial total sugar concentration was 6.55 per cent. After incomplete fermentation, the sugar content was 1.40 per cent, of which 0.25 per cent was glucose and 1.15 per cent was fructose. The selectivity constant was found to remain unchanged when sucrose was substituted for the sugar mixture and also when the proportion of glucose to fructose was changed.

On the basis of the theory of Michaelis and Menten (1913), the relative rates of reaction of two substrates competing for the same enzyme can be expressed by equation (2).

$$\frac{V_1K_2}{V_2K_1} = \frac{\log Y_0 - \log Y}{\log Z_0 - \log Z} \tag{2}$$

where K_1 and K_2 are the Michaelis constants expressed in terms of concentration, V_1 and V_2 are the maximum velocities for each substrate alone, and Y_0, Y, Z_0, and Z, have the same significance as in equation (1) (Haldane, 1930). From equations (1) and (2), $V_1K_2/V_2K_1 = K_{G/F}$, the selectivity constant; V_1 and K_1 refer to glucose, and V_2 and K_2 refer to fructose. Hopkins and Roberts (1935a) found that the maximal velocities of glucose and fructose fermentation are the same; hence, substituting the values of the Michaelis constants they determined gave $K_2/K_1 = 0.0225/0.01067 = 2.1 = K_{G/F}$ (selectivity constant). This is in close agreement with the value 2 calculated by Dawson (1932) from the earlier data of Hopkins (1931), and also agrees in general with the data of the other workers mentioned.

That these considerations must apply to the fermentation of sucrose in the dough is shown by the results of Rice (1938), who analyzed breads of various compositions and observed an excess of fructose over glucose when sucrose had been used in the formula. Examination of his data shows that a relatively high selectivity constant (about 4) appears to have functioned in his experiments, but it is conceivable that the difficulty of analyzing the complex mixture obtained from bread may be responsible for some discrepancy. Geddes and Winkler (1930) have shown that invert sugar (as in honey) is equivalent to sucrose. The rate of inversion of sucrose by bakers' yeast exceeds its rate of fermentation. Consequently, the addition of sucrose is equivalent to the addition of invert sugar.

Influence of Maltose. Although flour contains but little maltose, the formation of a dough initiates amylase activity and the maltose thus produced is often the principal sugar involved in the later stages of fermentation. The course of fermentation of pure maltose by bakers' yeast differs significantly from that of other sugars in the occurrence of a relatively long induction period (Blish and Sandstedt, 1937). The induction period of fermentation is somewhat loosely defined as the time that elapses before the system reaches the rapid or, in some cases, the maximal rate of fermentation. In solutions of pure maltose, bakers' yeast sometimes shows a very great induction period, so great, in fact, that the yeast appears to be incapable of fermenting the sugar. Blish and Sandstedt showed that certain naturally occurring substances found in flour and dried yeast have the property of shortening the induction period remarkably. The activator or activators were considered to be specific for maltose fermentation and were given the designation factor *M*. Schultz and Atkin (1939) and Leibowitz and Hestrin (1939) showed that the properties of factor *M* reside in either glucose or maltase. The flour contained glucose and the dried yeast contained maltase. The addition of a relatively small proportion of glucose sharply reduces the induction period in the fermentation of maltose. Figure 1, taken from Schultz and Atkin, illustrates this effect. The figure also

shows the effect of an extract of dried yeast. The influence of this extract was demonstrated to be due to the glucose liberated by maltase, contained in the extract, and not to the activity of any other substance present in the extract. The induction period may also be reduced by a small quantity of sucrose. Since flour contains at least 1 per cent of readily fermentable sugars, it is logical to suppose that there will be no induction period of consequence in a system composed of flour, yeast, maltose, and water.

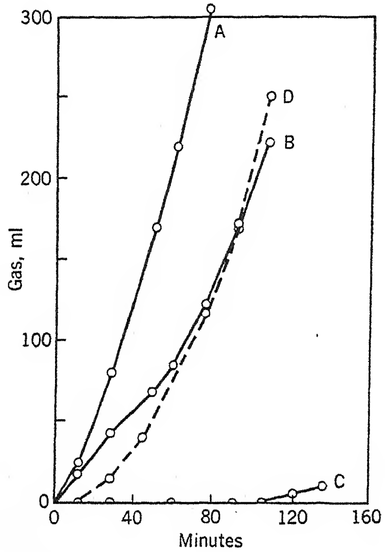

Fig. 1.—Influence of glucose on maltose fermentation (adapted from Schultz and Atkin, 1939). A, 3.0 g glucose; B, 2.8 g maltose plus 0.2 g glucose; C, 3.0 g maltose; D, 3.0 g maltose plus 40 ml maltase extract of yeast.

The peculiar circumstance that a yeast known to contain maltase is still unable to initiate maltose fermentation except after a lengthy induction period has caused some authors to support the direct maltose fermentation theory of Willstätter and Steibelt (1920). These authors, and Willstätter and Bamann (1926), Sobotka and Holzman (1934), Blish and Sandstedt (1937), and Leibowitz and Hestrin (1939, 1942), claim that yeast is capable of fermenting maltose without the intervention of maltase—that is, by direct fermentation. Willstätter and Steibelt inferred that fermentation of maltose must be direct from the disparity between the maltase content of certain yeasts and their ability to ferment maltose. They determined the maltase content of yeast by an autolytic method. But Krieble, Skau, and Lovering (1927) showed that, if the yeast is first dried and then extracted by an alkaline phosphate buffer solution, a large increase in the amount of maltase extracted is obtained. Maltase is notoriously unstable, and consequently it may be destroyed during autolysis of the yeast, as are many other of the yeast enzymes. On the other hand, maltase may be present in an inactive form in the original cell and be activated when the cell is dehydrated, but not when it autolyzes. In the absence of more exact information it is difficult to assess the potential maltase activity in the cell interior.

Willstätter and Bamann (1926) showed that the hydrogen-ion concentration optimum for maltase activity *in vitro* is near neutrality and that at values below pH 5.0 the enzyme is virtually inactive. These au-

thors, and also Sobotka and Holzman (1934), conclude that, when yeast is suspended in a medium of less than *p*H 5.0, the maltase is inactive *in vivo*. Consequently, they reason, a yeast which ferments maltose below *p*H 5.0 must do so without the aid of maltase. They likewise infer that a yeast grown at *p*H 2.5, for example, must inevitably be maltase-free. There is, however, no evidence whatsoever that the hydrogen-ion concentration of the interior of a living cell is the same as that of the surrounding medium. What evidence exists on this subject points to the contrary assumption; that is, the cell interior tends to maintain a relatively uniform hydrogen-ion concentration despite large changes in the acidity of the environment (Chambers, 1935).

Leibowitz and Hestrin (1939) consider that the behavior of yeast towards methyl-α-glucoside supports the case for direct fermentation. The argument for direct fermentation of maltose would be conclusive if a yeast species were to be found which is capable of fermenting maltose and is totally devoid of maltase activity. A more striking proof would be obtained if a yeast were found capable of fermenting maltose but not glucose.

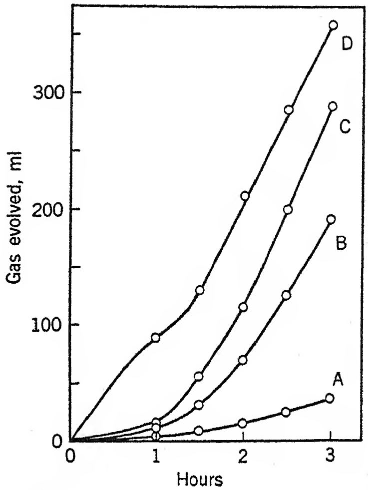

Fig. 2.—Influence of oxygen on maltose fermentation (adapted from Schultz, Atkin, and Frey, 1940). A, nitrogen atmosphere; B, air atmosphere; C, oxygen atmosphere; D, nitrogen atmosphere plus 0.4 g dextrose in solution.

Schultz, Atkin, and Frey (1940), and Kluyver and Custers (1940), observed another factor which has a potent influence on the induction period of maltose fermentation, namely, the effect of atmospheric oxygen. The normally long induction period is lengthened when an atmosphere of nitrogen is substituted for air. It is shorter in air and still shorter, although not absent, in pure oxygen. Figure 2 demonstrates this effect.

In the dough, maltose appears to be the last sugar to be fermented. Lanning (1936) and Larmour and Bergsteinsson (1936) have shown that sucrose, as well as glucose and fructose, are fermented before maltose in the dough. The latter authors showed that, when limited amounts of sucrose are added to the dough, the curve of fermentation exhibits two maxima. The initial rapid rate of sucrose fermentation falls off when the sucrose is exhausted, and then rises to a second maximum as the yeast commences the fermentation of maltose. These maxima are shown in the curve of instan-

taneous rate obtained by Landis and Frey (1943) with their fermentation rate meter (see Fig. 3). Larmour and Bergsteinsson showed that the position of the first maximum could be shifted to the right by further additions of sugar until at the 5 per cent sugar level it disappeared and the over-all curve showed only one maximum. Miller, Edgar, and Whiteside (1943) have also described the appearance of two maxima in the rate of gas production and indicate that the probable cause is closely associated with the supply of and demand on sugars within the dough.

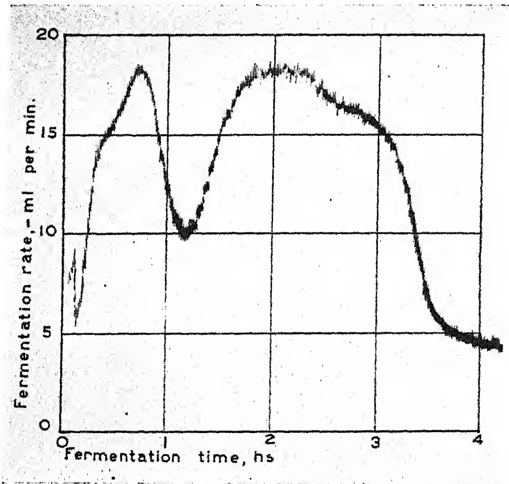

Fig. 3.—Sponge-type fermentation for a high-protein flour (Landis and Frey, 1943).

INFLUENCE OF NONSUGAR DOUGH CONSTITUENTS

Early attempts to reproduce the rate of dough fermentation in "synthetic" or artificial nutrient sugar solutions disclosed that fermentation in the dough proceeds at a much greater rate than it does in the best synthetic solution. Simpson (1936) compared flour dough with Hansen's yeast nutrient solution (Jørgensen, 1911) containing, in 60 ml, 0.5 g peptone, 0.15 g potassium phosphate (K_2HPO_4), and 0.10 g magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$); and with Slator's (1906, 1908) solution containing, in the same volume, 0.54 g asparagine, 0.18 g potassium phosphate, and 0.12 g magnesium sulfate. He found that, in Hansen's solution, yeast fermented at a higher rate than in Slator's, but the fermenting dough was still higher, to the extent of about 75 per cent. It should be noted that peptone cannot

be considered a synthetic ingredient in the ordinary sense, which implies a substance of known composition.

It is apparent, therefore, that flour contains a substance or substances which exert a powerful stimulating influence on the rate of alcoholic fermentation. The existence of such a substance in nature has long been suspected. Euler and Swartz (1924) gave the designation "factor Z" to this substance, which they found to be relatively widespread in extracts of plant and animal tissues. Schultz, Atkin, and Frey (1937, 1937a) discovered that vitamin B_1 (thiamine) has the properties of factor Z and is, in fact, indistinguishable from it. This makes possible a new attempt to reproduce the dough fermentation rate with a synthetic solution. The "complete" solution devised by Atkin, Schultz, and Frey (1945) consists of:

NaH_2PO_4, 0.06 g; $MgSO_4 \cdot 7\,H_2O$, 0.04 g; KCl, 0.016 g; asparagine, 0.2 g; thiamine, 0.08 mg; pyridoxine, 0.08 mg; niacin, 0.8 mg; and sodium citrate buffer ($M/3$) pH 5.5, 2.4 ml. In experiments with this solution (illustrated in Figures 4 to 9), 2 g of sucrose were fermented with 0.6 g of yeast in a total volume of 20 ml of solution maintained at 30°C.

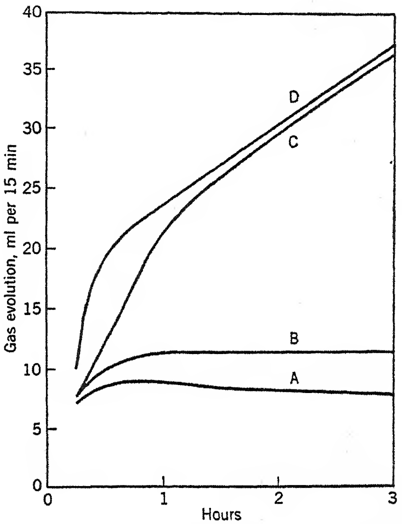

Fig. 4.—Comparison of gas evolution in flour paste and in synthetic solutions (Atkin, Schultz, and Frey, 1945). A, control (sucrose only); B, 0.2 g NaCl and 0.2 g asparagine; C, 20 g flour, 0.2 g NaCl, and 0.2 g asparagine; D, "complete" medium.

Figure 4 (Atkin, Schultz, and Frey, 1945) shows that success apparently has been achieved in formulating a synthetic medium in which yeast ferments with a rate equal to that in a dough. Under the experimental conditions employed, there is a constant acceleration of the rate of fermentation over the 3-hour period.

The so-called "complete" synthetic solution contains a number of substances, all of which are known to occur in flour (Bailey, 1944). The significance of each of these factors for alcoholic fermentation by the living yeast cell has been investigated. Potassium, magnesium, ammonium, sulfate, and phosphate ions appear to be the principal inorganic ions capable of markedly influencing the rate of fermentation. Because yeast is able to use both organic and inorganic nitrogen (from certain compounds), the effect of ammonium ions and amino nitrogen will be discussed separately.

Phosphate and Sulfate. Phosphate has long been known to be concerned with the internal mechanism of alcoholic fermentation (Harden, 1932). It has been employed in yeast nutrient solutions from early times (Jørgensen, 1911). It enters into the carboxylase enzyme system in the form of cocarboxylase, which is diphosphothiamine (Lohmann and Schuster, 1938). It is common practice to add phosphates to sugar-rich materials

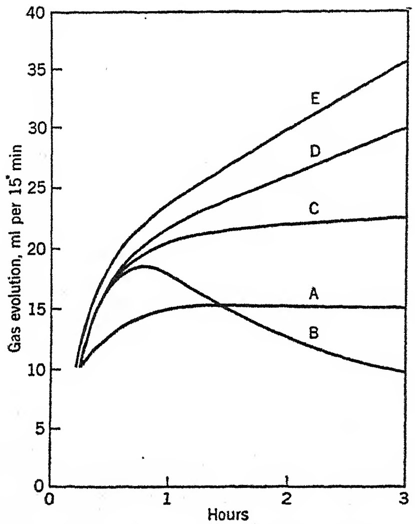

Fig. 5.—Effect of potassium chloride, magnesium sulfate, and sodium phosphate on rate of fermentation of sucrose (Atkin, Schultz, and Frey, 1945). A, "complete" medium minus NaH_2PO_4; B, "complete" medium minus $MgSO_4 \cdot 7\,H_2O$ but plus 0.04 g Na_2SO_4; C, "complete" medium minus $MgSO_4 \cdot 7\,H_2O$ but plus 0.04 g magnesium lactate; D, "complete" medium minus KCl; E, "complete" medium.

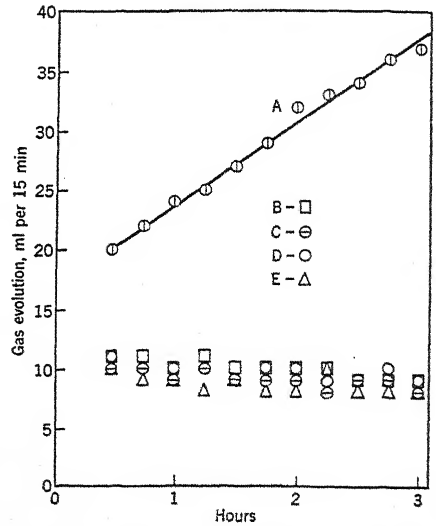

Fig 6.—Effect of potassium chloride, sodium phosphate, and magnesium sulfate on rate of fermentation of sucrose (Atkin, Schultz, and Frey, 1945). A, "complete" medium added; B, no addition except 0.06 g NaH_2PO_4; C, no addition except 0.04 g $MgSO_4 \cdot 7\,H_2O$; D, no addition except 0.016 g KCl; E, no addition.

such as molasses prior to their fermentation. Larmour and Bergsteinsson (1936), however, state that the phosphate ion has little, if any, specific effect on gas production. Their experiments were made with fermenting doughs and, as Swanson (1912) has shown, there is a significant quantity of water-extractable phosphorus in patent flour. Therefore, it must be assumed that if phosphate does play a role in yeast fermentation in the dough, the flour used by Larmour and Bergsteinsson contained an excess. The nature of the soluble phosphate in flour is not exactly known, but Swanson suggests that at least a part is monopotassium phosphate. A good part of

the phosphorus of wheat exists in organic combination either as phytates or phospholipides (Bailey, 1944). Widdowson (1941) has reported that 85 per cent of the phytic acid originally contained in white flour is destroyed in the preparation of yeast bread. It is possible that destruction of phytic acid coincides with the liberation of soluble phosphate.

The proportion of the phosphate of flour which is separated from the organic moieties by hydrolysis during the doughing process is at present undetermined.

Examination of Figures 5 and 6, taken from Atkin, Schultz, and Frey (1945), discloses that, although phosphate alone has an insignificant effect upon the rate of fermentation, it is essential for the elevated rate of fermentation observed in doughs. In the absence of phosphate, the rate of fermentation in the otherwise complete mixture is scarcely different from that in a simple sugar–water–yeast mixture. It is therefore probable that the phosphate content of flour is ordinarily sufficient to maintain yeast at its maximal fermentation rate, provided all other factors are present in adequate amount.

Sulfur, like phosphorus, is an important constitutent of cell protoplasm. However, it has not yet been reported to be directly associated with alcoholic fermentation. In the form of sulfate it is available to the cell for the synthesis of the sulfur-containing amino acids, cystine and methionine and the tripeptide glutathione. Magnesium sulfate is a constituent of the media of both Hansen and Slator. Although there is sulfur in the ash of white flour, there is no evidence concerning the form of its compounds. Neither is there any data on the proportion which is water-soluble. The data of Atkin, Schultz, and Frey (Figs. 5 and 6) show the influence of sulfate upon the fermentation rate. In the absence of sulfate, the rate obtained in the otherwise complete mixture is somewhat higher than without phosphate, but here also acceleration is lacking. Magnesium sulfate alone is without significant effect on fermentation rate. The separate omission of sulfate was accomplished by the substitution of magnesium lactate for magnesium sulfate. A separate test showed that a mixture of magnesium lactate and sodium sulfate is equivalent to a corresponding amount of magnesium sulfate. It seems probable from these data that sulfate, in adequate amount, is present in white flour.

Magnesium and Potassium. Magnesium has also been employed in yeast nutrient solutions since early times (Jørgensen, 1911). It is concerned with the internal mechanism of alcoholic fermentation (Kossel, 1942; Green, Herbert, and Subrahmanyan, 1941). Harding and Dysterheft (1927) found 0.0613 per cent of magnesium in clear flour. Here, as with phosphorus, the nature of the soluble magnesium compounds is not known. Figures 5 and 6 show that it is possible that magnesium plays an important role in

dough fermentation. The specific effect of magnesium is demonstrated by the substitution of sodium sulfate for magnesium sulfate in the "complete" mixture. It is apparent that, although magnesium does not by itself stimulate fermentation, it is essential for the maintenance of the accelerated fermentation rate observed in the "complete" medium.

The exact role of potassium in fermentation is not known, but its importance in accelerated fermentation can be judged from Figures 5 and 6. As with phosphorus and magnesium, there is evidence that flour contains significant quantities of potassium, and hence conceivably could make available adequate amounts for the nutrition of the yeast. Lasnitzki and Szörényi (1935) observed that potassium increased the rate of fermentation of bakers' yeast.

Whether all flours contain available phosphates, sulfate, magnesium, and potassium in sufficient amount to prevent these factors from ever becoming a limiting factor in dough fermentation is not known. From the whole of the present discussion it is apparent that any single one of these factors essential for fermentation might be present in a flour in submaximal amount for maximal fermentation with 3 per cent yeast, but that such deficiency would be unnoticed with a lower percentage of yeast or in a dough which has not been supplied with optimal amounts of other necessary factors, *e.g.*, amino nitrogen.

Ammonium and Amino Nitrogen. Willard and Swanson (1913) found that the rate of fermentation of doughs was increased by the addition of peptones, certain amino acids, and ammonium salts. Ammonium chloride gave a marked increase, but ammonium phosphate (dibasic) was reported to be without action. These authors also found that an extract of bran or wheat scourings had a stimulatory effect on dough fermentation.

In 1915, Kohman, Hoffman, and Blake obtained a patent covering the addition of ammonium salts to the dough for the purpose of enhancing fermentation activity. Although ammonium salts or assimilable amino nitrogen compounds had been employed in yeast nutrient solutions earlier, it was not widely recognized for many years that such substances are fermentation accelerators. Zeller (1926) observed stimulation of fermentation by a large number of ammonium salts. Euler and Larsson (1934) observed that the activity of one of their factor *Z* preparations was higher when it contained ammonium ions. When tested separately, ammonium sulfate gave some stimulation, but much less than their concentrate of *Z* factor. Larmour and Bergsteinsson (1936) found that ammonium salts exert a specific stimulation in dough fermentation, particularly in the latter stages, that is, after the first hour or two.

Sandstedt and Blish (1938) observed that flours differed widely in the third hour, or "proof time" fermentation rate, when tested in the fol-

lowing formula: 20 g of flour, 0.8 g of maltose, 20 ml of water, and 0.6 g of yeast. They ascribed the differences to a variation in factor M content, factor M being a specific catalyst for maltose fermentation. Schultz, Atkin, and Frey (1939b) suggested that the difference between flours might be due to their content of amino nitrogen. They showed that the addition of various amino acids increased the third-hour fermentation rate to a significant extent, and tended to make the rates of different flours alike. Ofelt and

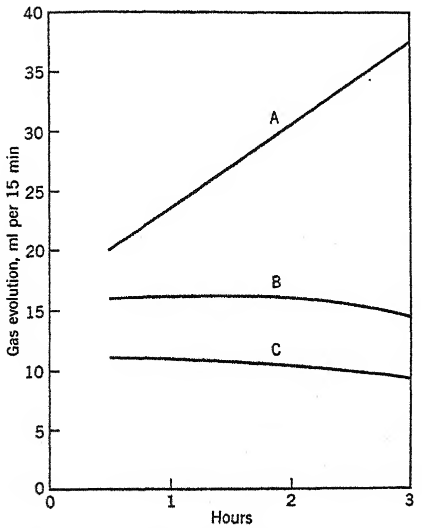

Fig. 7.—Influence of asparagine on fermentation of sucrose (Atkin, Schultz, and Frey, 1945). A, "complete" medium added; B, "complete" medium minus asparagine; C, no addition except 0.2 g asparagine.

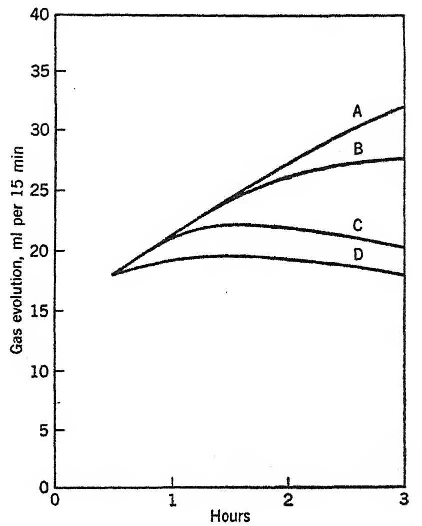

Fig. 8.—Influence of concentration of asparagine on rate of fermentation of sucrose in "complete" medium except for amount of asparagine (Atkin, Schultz, and Frey, 1945). A, 80 mg asparagine; B, 40 mg; C, 20 mg; D, 10 mg.

Sandstedt (1940) found that the third-hour proof rate of a series of flours increased in almost linear relationship with increasing protein content. Hydrolyzed gluten was added to a number of samples of flour of widely different protein contents, and this brought all flours to a nearly uniform rate of third-hour gas production. Ammonium salts accomplish the same thing, but it was found that *l*-asparagine did not succeed in bringing all rates to the same level.

Yeast is capable of utilizing nitrogen from other sources besides ammonium salts (Thorne, 1933). The amide nitrogen of asparagine is readily available to yeast, probably because yeast contains an asparaginase which splits off the ammonia from the amide group (Grassmann and Mayr, 1933).

Figure 7 shows the relation of nitrogen to maximal fermentation; it is taken from Atkin, Schultz, and Frey (1945). As with phosphate and certain other compounds of the "complete" mixture, the addition of asparagine to a simple sugar–water solution is without significant effect. The addition of all of the other factors of the "complete" medium, except asparagine, permits fermentation to proceed at a slightly elevated but uniform rate. The completion of the mixture by addition of asparagine shows the accelreated rate characteristic of the dough fermentation. Equivalent results are obtained with the use of ammonium sulfate as a source of nitrogen.

As Larmour and Bergsteinsson (1936) have observed, the effect of ammonium salts added to the dough appears in the latter stages of fermentation. The reason for the apparent delayed action becomes clear when one considers the data of Figure 8 from Atkin, Schultz, and Frey. Here, the effect of graded amounts of asparagine is shown. With moderate amounts of asparagine the initial rates are all alike, but the curves level off one after the other as the nitrogen becomes the limiting factor. If a flour contains available nitrogen, equivalent to one of the intermediate levels, it is apparent that the addition of further nitrogen will affect the rate of fermentation only after the passage of time.

Sources of Assimilable Nitrogen in the Dough. There are three general sources of yeast-assimilable nitrogen in the dough: first, the water-soluble nitrogen compounds originally present in the flour; second, the soluble amino acids and other forms of available nitrogen which result from the activities of proteolytic enzymes of the flour and other ingredients in the presence of insoluble or complex nitrogenous compounds; and third, nitrogen-containing dough ingredients other than flour.

The soluble nitrogen initially present in the dough has been determined by Swanson (1912), who extracted flour with a large volume of water at room temperature for 1 hour, and then filtered. To the filtrate he added phosphotungstic acid and filtered again. Nitrogen was determined in the filtrate, calculated as protein, and expressed as per cent of soluble amino compounds. Swanson obtained 0.53 per cent in wheat and 0.162 per cent in a 70 per cent patent flour. Blish (1918) reported that "normal patent flour contains but about 2 mg of amino-acid nitrogen for every 100 gm of flour, and about three times as much nitrogen in free acid amide form." From the results of Ofelt and Sandstedt (1940), it may be inferred that flours differ widely in soluble nitrogen, and that the amount that is soluble is proportional to total protein or nitrogen content.

The relation of proteolytic activity to baking technology is the subject of another chapter, but it is apparent from the work of Freilich and Frey (1939, 1941, 1943) that proteolytic activity in doughs, whether from original enzyme content or added enzyme (papain), can produce significant

amounts of yeast-assimilable formol-titratable nitrogen. These authors also show that mixing the dough in oxygen or nitrogen influences proteolytic activity, and it may be supposed (see above) that corresponding secondary effects on fermentation rates are possible.

Ammonium salts have been added to doughs for many years. They are frequently found in so-called dough conditioners, dough improvers, or yeast foods, often mixed with other substances such as bromates, which are added for different purposes. An early patent for a dough ingredient issued to Kohman, Hoffman, and Godfrey (1915) specifies the following mixture to be added to 100 pounds of flour; 0.64 oz of ammonium chloride, 1.76 oz of calcium sulfate, 0.0176 oz of potassium bromate, and 2.9 oz of flour.

Yeast-assimilable nitrogen is frequently added in other forms. One example is milk solids. Munz and Bailey (1936) found that "... certain milk constituents tend to accelerate yeast fermentation in bread doughs and particularly when an abundance of fermentable sugars is present." From the foregoing it may be surmised that the increased rate of fermentation was due to yeast-assimilable nitrogen in the milk. St. John and Bailey (1929) had previously found that production of total gaseous carbon dioxide in yeast-leavened doughs was increased when dry skim milk was superimposed upon the control formula. Skovholt and Bailey (1937) also noted that milk solids accelerated gas production in doughs when sufficient sugar was present. These authors believe that a part of the acceleration is probably due to the effect of milk solids upon hydrogen-ion concentration. It will be appreciated from the foregoing, however, that milk solids contain sufficient soluble nitrogen to explain the greater part of the fermentation accelerating effect. Kohman, Godfrey, and Ashe (1917) found that, after the digestion of nitrogenous material to convert it to a form in which it is rich in nitrogen available as a yeast food, the product showed an enhanced acceleration of fermentation. They recommend, for example, the use of certain cheeses.

Vitamins. Euler and Swartz (1924) are responsible for initiating the intensive study of an organic substance, occurring widely in nature, which is capable of causing a marked stimulation of alcoholic fermentation by living yeast. They called this substance "factor *Z*." Euler and his co-workers devoted much work to the study of the nature of the elusive substance (Euler and Larsson, 1934). Schultz, Atkin, and Frey (1937, 1937a) observed that vitamin B_1, later designated thiamine, had the properties of factor *Z*. When the activity of the pyrimidine moiety of the thiamine molecule was shown (Schultz, Atkin, and Frey, 1937a), the identity of factor *Z* and thiamine (or the specific amino pyrimidine) became even more certain. Vitamin B_1 activity was known to be alkali labile. Euler's school had noted that alkaline hydrolysis did not destroy *Z* factor activity, and for this reason they

did not associate vitamin B_1 and *Z* factor. The amino pyrimidine produced by hydrolysis of thiamine retains the fermentation accelerating activity, although inactive as a vitamin.

Thiamine is always present in bread flour. Although a large proportion of the thiamine of the wheat berry is removed in the course of the milling process which produces white flour (Schultz, Atkin, and Frey, 1939a), the amount retained is sufficient to exert maximal fermentation acceleration in most cases (Schultz, Atkin, and Frey, 1939b). In the latter publication, a flour is described (first middlings), which was so low in thiamine that addition of the vitamin caused a significant increase in the third-hour fermentation rate. Although thiamine deficiency does not appear to be a normal occurrence in breadmaking, it must be recognized that thiamine is an essential factor in the mechanism of alcoholic fermentation in the dough. Reference to Figure 9, from Atkin, Schultz, and Frey (1945), shows that thiamine is without significant effect upon the fermentation rate when added to a sugar–water solution, but it is essential for the development of the high accelerated rate characteristic of dough fermentation.

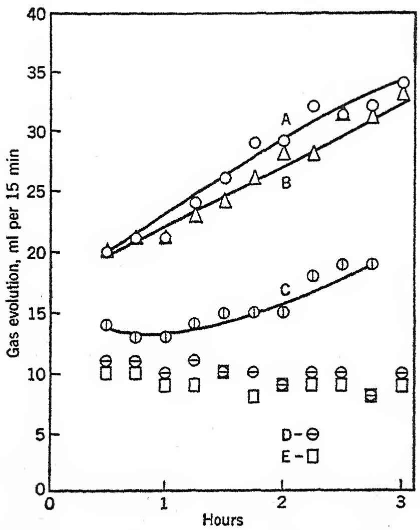

Fig. 9.—Influence of thiamine and pyridoxine on fermentation of sucrose (Atkin, Schultz, and Frey, 1945). A, "complete" medium; B, "complete" medium minus pyridoxine; C, "complete" medium minus thiamine; D, no addition except 0.08 mg thiamine; E, no addition except 0.08 mg pyridoxine.

Pyridoxine (vitamin B_6) has a much smaller effect upon fermentation (Schultz, Atkin, and Frey, 1939). Like thiamine, it is present in white flour in amounts adequate to promote maximal fermentation (Atkin, Schultz, Williams, and Frey, 1943). The influence of this vitamin on maximal fermentation is shown in Figure 9. Niacin (nicotinic acid) has an even smaller effect than pyridoxine (Schultz, Atkin, and Frey, 1938), but it is included in the "complete" medium as a precautionary measure.

Toxic Substance. It has been known for a long time that in cereals some substances are present that have an inhibitory effect on fermentation. Baker and Hulton (1910) observed that the effect is more pronounced with distillers' yeast than with brewers' yeast. Lecourt (1927) gives an excellent summary of earlier work. The substance may be extracted from flour if a

small quantity of water is used and filtration is rapid. It appears to be associated with the gluten fraction of flour. An extract so prepared actually kills the yeast cells and appears to be adsorbed on the dead cells. The rapid action of the poison does not manifest itself in the absence of fermentable sugar, and when it does its activity appears to be a function of sugar concentration. All salts have the power of neutralizing or preventing the action of the toxic agent, if employed in sufficient concentration. Salts differ in their antitoxic power; for example 0.02 per cent of calcium chloride has an action equivalent to 1.0 per cent of sodium chloride.

According to Stuart and Harris (1942), the protein isolated from wheat and crystallized by Balls, Hale, and Harris (1942) is in all probability the substance responsible for the toxicity of wheat flour. Balls and coworkers isolated a crystalline substance of protein-like nature from the petroleum ether extract of wheat flour. Analytical examination has shown that the substance consists mainly of amino acid residues. The crystals are the hydrochloride of a basic substance, and the compound has a minimum molecular weight of 6000 and a probable molecular weight of double that value. In the grain and in flour, the material exists in the reduced form as a sulfhydryl compound. It is believed to occur in combination with a phosphorus-bearing lipide, and hence may be classed with the lipoproteins. The same authors, in further work (Balls, Hale, and Harris, 1942a), find that the sulfur content is among the highest known for proteins. This sulfur, present as cystine, may be reversibly oxidized and reduced. The name "purothionin," derived from the Greek for wheat and sulfur, is proposed for the substance isolated.

Stuart and Harris (1942) tested the compound against *Saccharomyces cerevisiae* and found it to possess a marked fungicidal activity, which brings about the death of yeast cells in concentrations of 0.005 mg per ml and higher. No lysis of the yeast cells was observed. At lower concentrations the multiplication of yeast is prevented. Balls and Harris (1944) studied the effect of the substance (now called a protamine) on the fermentation of wheat mashes. They found that either heating or proteolysis destroyed the toxicity. The substance also tends to undergo an apparent "alkaline" denaturation, even at hydrogen-ion concentration slightly below neutrality. Its activity is completely destroyed by the pressure cooking incident to mashing the grain preparatory to saccharification and fermentation.

The doughing process is apparently conducted under conditions ideal for the operation of this toxic factor, yet obviously yeast activity is not repressed. Certain unpublished data of Schultz, Atkin, and Frey may be used to explain this apparent anomaly. The following figures show how flour affects the gas evolved at the end of 3 hours at 30°C

by 2 g of yeast in 20 ml of an aqueous solution containing 2 g of sucrose:

Flour, *g*	0	1	2	5	10	10 + 0.2 g salt
Gas, *ml*	94	59	46	90	143	226

Although flour contains substances capable of greatly stimulating the fermentation rate (see above), the first additions of flour cause a marked decrease in rate. The rate gradually increases, but at 10 g of flour (200 per cent absorption) the toxic factor is still operating, as evidenced by the marked stimulation produced by the addition of salt. Other experiments show that at 100 per cent absorption (20 g of flour) the toxic factor may still be effective because the addition of salt raises the third-hour gassing rate. The activity of the toxic factor tends to be minimized as dough conditions are approached (70 per cent absorption), and this may be explained on the basis of increased salt concentration due to the soluble solids of the flour itself. The effect of increasing amounts of salt on the inhibition produced by 5 g of flour (temperature, 30°C; yeast, 0.6 g; sucrose, 2 g; volume, 20 ml) is shown by the following data:

Salt, *g*	0	0.025	0.05	0.1	0.2
Gas, *ml*	69	107	155	199	201

It is apparent, however, that the ordinary dough mixture is close to the flour–water ratio at which the toxic factor may be effective. Experiments made with flour suspensions or pastes are always subject to suspicion unless sufficient salt is added. As regards dough, it is not improbable that flours may vary in their content of the toxic factor; and, consequently, dough tests conducted without salt may be in error. The susceptibility of various yeasts may vary, depending upon the strain or method of culture (Lecourt, 1927). Yeast concentration may also be a factor. Further study of the toxic factor of wheat flour, as it affects bread manufacture, seems to be desirable.

CHANGES IN THE YEAST POPULATION OF THE DOUGH

If the number of yeast cells changes during the life of the dough as a consequence of growth, it is apparent that the rate of fermentation may be affected. Determination of the yeast population is ordinarily made by counting the cells in the same manner as blood counts are made, that is, using a hemocytometer. Turley (1924) digested the dough with pepsin–hydrochloric acid, prior to counting, in order to free the cells from the gluten. Simpson (1936) used a similar procedure, but followed the first

digestion with a treatment designed to disintegrate the starch granules—that is, heating to 90°C, followed by digestion with diastatic malt extract at 65°. The cells are stained with methylene blue. Hoffman, Schweitzer, and Dalby (1941) stopped yeast growth with chloroform, washed the gluten relatively free of yeast cells and starch, and then disintegrated the gluten with dilute hydrochloric acid. The mixture, including the disintegrated gluten, is stained with carbol fuchsin which stains the yeast cells and gluten particles a dark red color and the starch granules a pale pink. The authors claim that it is easy to differentiate the yeast cells from the gluten particles by a difference in shape.

Simpson (1936a) observed that, when the yeast in the formula is less than 2 per cent based on the flour, a significant increase in cell number takes place in the dough. Hoffman, Schweitzer, and Dalby (1941a) find some growth at the 2 per cent yeast level after 6 hours. With low yeast levels, they find that ammonium chloride and calcium sulfate addition causes an increase in yeast growth. Under the conditions of their test they find an apparent relation between yeast-cell count and loaf volume. The highest loaf volume was found at 1.75 per cent original yeast content. At this level the increase in cell count was also maximal.

The relationship between yeast growth in the dough and the rate of fermentation is not clear. The rate of gas evolution from a dough characteristically shows an increase with time. This increase is noted at levels higher than 2 per cent (that is, 3 per cent), as shown elsewhere in this chapter (page 335). If yeast growth is absent or even limited at the 2 per cent level, it is apparent that the increased rate of fermentation cannot be due to an increase in the number of yeast cells. Moreover, it would seem that, even when yeast growth does occur, not all of the increased activity is due to an increase in numbers. No attempt has yet been made to obtain a measure of the size of the cells in the dough nor the weight of yeast solids in the dough; obviously these are very difficult measurements to make. It cannot be disputed that an increase in cell radius would cause a significant increase in cell surface. The larger surface could conceivably be responsible for an increased fermentation rate.

Ideally it would be desirable to separate the two factors, growth and increase in rate; but it appears that practical dough conditions are conducive to both growth and fermentation, and consequently the two are very difficult to separate. It is to be remembered, however, that it is the objective of baking technology to utilize gas production in the dough, regardless of the source. In this sense the distinction becomes an academic one. In the numerous references to experiments dealing with gas production in solutions, suspensions, and doughs, one finds no attention paid to possible changes in yeast cell number.

FERMENTATION IN THE DOUGH

From the foregoing it is evident that a dough may be described as a favorable although not always ideal environment for alcoholic fermentation by living yeast. In a dough, the yeast is suspended in an aqueous medium which contains fermentable sugars, available nitrogen, phosphates, sulfates, magnesium, potassium, thiamine, and pyridoxine, all of which appear to be essential for the optimum fermentation. All of these factors are not always present in sufficient amount to promote maximal fermentation activity. Occasionally, sugar and salt may be present in amounts high enough to inhibit fermentation to some extent.

When all the known fermentation factors are present in a mixture in optimal amount, the rate of gas production is constantly accelerated. This type of gas production rarely occurs under practical dough conditions. In virtually all cases the acceleration is arrested after a certain period, at which time the rate remains relatively constant or perhaps falls to a lower level, after which it may suddenly rise again. It is probable that the decline from maximum rate is caused by the gradual exhaustion of one of the essential fermentation factors. As shown earlier in this chapter, the two factors that are most frequently deficient are fermentable sugar and available nitrogen. Lack of fermentable sugar first makes its appearance in the sponge fermentation (see Fig. 3, page 334). A typical sponge dough contains little or no added fermentable sugar but only that originally contained in the flour. When this type of dough is mixed, the yeast first ferments the readily available sugar (glucose and sucrose), and when this is exhausted there is an abrupt fall in fermentation rate; but after a short time the yeast goes over to maltose, and the rate rises with the same abruptness. Thus the change in fermentation rate is due to the momentary lack of fermentable sugar. Strictly speaking, there is no true lack of fermentable sugar during this interval but, momentarily, the maltose that is present is unfermentable or only slowly fermented by the bakers' yeast.

As the sponge fermentation is continued, another abrupt fall in fermentation rate eventually occurs (see Fig. 3). This fall results from the near exhaustion of all fermentable sugar. Fermentation proceeds at a low level, sometimes called the diastatic level, which means that the rate of fermentation is controlled solely by the amount of sugar produced by the diastatic enzymes of the dough. It is fairly obvious that the time at which this final fall in fermentation rate occurs will be determined by a number of factors, which are temperature, yeast concentration, initial sugar content of the dough, and the sugar-producing ability of the enzyme–substrate system. If any three of these four factors are kept constant, the remaining one will determine the duration of active fermentation.

When the sponge is mixed with the ingredients needed to form the dough (more flour, and water, sugar, salt, milk solids, and shortening), the elevated rate of fermentation is resumed. Bread must be made from this dough before sugar exhaustion again becomes a limiting factor. Since there is usually ample sugar in the dough stage, the factor that appears to control the rate of fermentation attainable is the available nitrogen. All other things being equal, the maximum rate of fermentation during this period will depend on the supply of available nitrogen at this time.

In the straight-dough process, all of the dough ingredients are mixed together at one time. In this case, sufficient sugar is usually present (for example, 3 to 6 per cent based on the flour) to eliminate the first drop in fermentation rate, which coincides with the change-over from sucrose to maltose. Otherwise, the same general considerations apply to the straight dough as apply to the sponge and dough process. Mention of specific quantities and proportions is omitted because there are many variations possible in bread-dough formulas, and the choice of any single formula might readily lead to an inaccurate impression of the problem as a whole.

Little has been said up to this point about the final objective of yeast fermentation in a bread dough. This objective may be simply stated as a well-leavened loaf of bread produced in a regular, controllable manner. In a modern bakery, regularity of mixing times, dough times, and proof times may be of crucial importance. For this, the baker depends upon the miller who must blend his flour to avoid erratic changes in composition, and upon the yeast manufacturer who must prepare and deliver his highly perishable product and at the same time maintain a high degree of uniformity in the action of the yeast in a dough.

The production of a well-leavened loaf is the problem of the bakery engineer. When the dough has finally been molded, panned, and placed in the proof box, it must be fermenting at a relatively rapid rate. When the dough volume reaches a predetermined value (usually the top of the baking pan), it is placed in the oven. Here the temperature of the dough rises rapidly and all the enzymatic processes are greatly accelerated. As each critical temperature is reached, the enzyme reactions cease one after the other, and finally the dough is coagulated. A short time later the finished bread is taken from the oven. Between the time the dough is placed in the oven and the time that the bread is fully coagulated, or fixed in volume, a great increase in volume occurs. Part of this increase is caused by the thermal expansion of the gases and vapors of the dough, but a very significant part is due to fermentation, which continues until the critical temperature is reached. It follows from this that, other things being equal, the final volume of the loaf will depend upon the rate of fermentation of the dough when it is placed in the oven. The fundamental problem, therefore, is to design

the dough procedure so as to bring the dough to the oven when the rate of fermentation is most suitable.

BIBLIOGRAPHY

ATKIN, L., SCHULTZ, A. S., and FREY, C. N. (1945). Influence of dough constituents on fermentation. *Cereal Chem.* 22:321–333.

——, ——, WILLIAMS, W. L., and FREY, C. N. (1943). Yeast microbiological methods for determination of vitamins. Pyridoxine. *Ind. Eng. Chem., Anal. Ed.* 15:141–144.

BAILEY, C. H. (1944). *Constituents of Wheat and Wheat Products.* Reinhold, New York.

—— and SHERWOOD, R. C. (1923). The march of hydrogen-ion concentration in bread doughs. *Ind. Eng. Chem.* 15:624–627.

BAKER, J. L., and HULTON, H. F. E. (1910). The toxins in cereals. *J. Inst. Brewing* 16:395–409.

BALLS, A. K., HALE, W. S., and HARRIS, T. H. (1942). A crystalline protein obtained from a lipoprotein of wheat flour. *Cereal Chem.* 19:279–288.

——, ——, and —— (1942a). Further observations on a crystalline wheat protein. *Cereal Chem.* 19:840–844.

—— and HARRIS, T. H. (1944). The inhibitory effect of a protamine from wheat flour on the fermentation of wheat mashes. *Cereal Chem.* 21:74–79.

BLISH, M. J. (1918). A study of the non-protein nitrogen of wheat flour. *J. Biol. Chem.* 33:551–559.

—— and HUGHES, R. C. (1932). Some effects of varying sugar concentration in bread dough on fermentation by-products and fermentation tolerance. *Cereal Chem.* 9:331–356.

—— and SANDSTEDT, R. M. (1937). Biocatalytic activators specific for the yeast fermentation of maltose. *J. Biol. Chem.* 118:765–780.

——, ——, and ASTLEFORD, G. R. (1932). Sugars, diastatic activity, and "gassing" power in flour. *Cereal Chem.* 9:378–393.

CHAMBERS, R. (1935). The living cell. Chapter I. In B. Harrow and C. P. Sherwin, *Textbook of Biochemistry.* Saunders, Philadelphia.

COLIN, H., and BELVAL, H. (1935). Les glucides de la farine et de la pâte. *Compt. rend.* 200:2032–2034.

—— and —— (1935a). La lévosine des grains et des farines. *Bull. soc. chim. biol.* 17: 1040–1044.

DAWSON, E. R. (1932). The selective fermentation of glucose and fructose by yeast. *Biochem. J.* 26:531–535.

EKSTEDT, H. G. (1938). Sponge and dough temperatures. *Am. Soc. Bakery Engrs. Proc. 15th Ann. Meeting*:35–45.

EULER, H. v., and LARSSON, H. (1934). Bios and Factor Z. *Z. physiol. Chem.* 223: 189–206.

—— and SWARTZ, O. (1924). Über den Zusammenhang der wasserlöslichen Wachstumsfaktoren mit Aktivatoren des Zuckerabbaues und über einen thermostabilen Biokatalysator in der Hefe. I. *Z. physiol. Chem.* 140:146–163.

FISHER, E. A., and HALTON, P. (1929). Relation of hydrogen-ion concentration and buffer value to the baking quality of flour. Part I. *Cereal Chem.* 6:18–33.

—— and —— (1929a). The relation of hydrogen-ion concentration and buffer value to the baking quality of flour. Part II. *Cereal Chem.* 6:97–115.

—— and —— (1937). Studies on test baking. I. The technique and some factors affecting fermentation. *Cereal Chem.* 14:349–372.

FREILICH, J., and FREY, C. N. (1939). Dough oxidation and mixing studies. I. The action of potassium bromate in dough. II. Effects of remixing after fermentation. III. The effects of proteases and reducing substances on dough when mixed in oxygen. *Cereal Chem.* 16:485–512.

FREILICH, J., and FREY, C. N. (1941). Dough oxidation and mixing studies. IV. Effects of oxygen and potassium bromate in sponge doughs. *Cereal Chem.* 18:78–86.

—— and —— (1943). Dough oxidation and mixing studies. V. Correlation between protease activity, reducing matter, and oxidizing effects in dough. *Cereal Chem.* 20:301–310.

GEDDES, W. F., and WINKLER, C. A. (1930). A study of the relative value of honey and sucrose in bread manufacture. *Can. J. Research* 3:543–559.

GEOFFROY, R. (1935). Action de la levure haute sur la lévosine en solution et dans les pâtes de boulangerie. *Bull. soc. chim. biol.* 17:848–851.

GRASSMANN, W., and MAYR, O. (1933). Zur Kenntnis der Hefeasparaginase. *Z. physiol. Chem.* 214:185–210.

GREEN, D. E., HERBERT, D., and SUBRAHMANYAN, V. (1941). Carboxylase. *J. Biol. Chem.* 138:327–339.

GUILLEMET, R. (1935). La fermentation catalysée de quelques fructohalosides. *Compt. rend.* 201:1517–1519.

—— and SCHELL, C. (1934). Remarques concernant l'influence de la température et de la pression sur la fermentation alcoolique par la levure, dans les solutions de farine de blé. *Compt. rend. soc. biol.* 116:1399–1401.

HALDANE, J. B. S. (1930). *Enzymes.* Longmans, Green, London.

HARDEN, A. (1932). *Alcoholic Fermentation.* 4th ed. Longmans, Green, New York.

HARDING, E. P., and DYSTERHEFT, G. (1927). A determination of iron, calcium, magnesium, phosphorous, ash, and protein in hard spring wheat and in the flour streams representing the largest volume of flour produced in its milling. *Cereal Chem.* 4:47–57.

HEALD, W. L. (1932). Some factors which affect gas production during dough fermentation. *Cereal Chem.* 9:603–616.

HOFFMAN, C., SCHWEITZER, T. R., and DALBY, G. (1941). The counting of yeast cells in bread doughs. *Cereal Chem.* 18:337–342.

——, ——, and —— (1941a). Factors affecting the growth of yeast in fermenting doughs. *Cereal Chem.* 18:342–349.

HOPKINS, R. H. (1928). The selective fermentation of glucose and fructose by brewer's yeast. *Biochem. J.* 22:1145–1156.

—— (1931). Selective fermentation. Alcoholic fermentation of mixtures of glucose and fructose by brewer's and Sauterne yeasts. *Biochem. J.* 25:245–255.

—— and ROBERTS, R. H. (1935). The kinetics of alcoholic fermentation of sugars by brewer's yeast. I. Effect of concentrations of yeast and sugar. *Biochem. J.* 29:919–930.

—— and —— (1935a). The kinetics of alcoholic fermentation of sugars by brewer's yeast. II. The relative rates of fermentation of glucose and fructose. *Biochem. J.* 29:931–936.

JESSEN-HANSEN, H. (1911). Studies on wheat flour. I. Influence of the concentration of hydrogen ions on the baking value of flour. *Compt. rend. trav. lab. Carlsberg* 10:170–206.

JØRGENSEN, A. P. C. (1911). *Micro-Organisms and Fermentation.* 5th ed. Griffin, London.

JOHNSON, A. H. (1925). Identification and estimation of the organic acids produced during bread dough and cracker dough fermentation. *Cereal Chem.* 2:345–364.

—— and BAILEY, C. H. (1924). A physico-chemical study of cracker dough fermentation. *Cereal Chem.* 1:327–410.

KLUYVER, A. J. (1914). Biochemische Suikerbepalingen. *Dissertation*, Delft.

—— and CUSTERS, M. T. J. (1940). The suitability of disaccharides as respiration and assimilation substrates for yeasts which do not ferment these sugars. *Antonie van Leeuwenhoek J. Microbiol. Serol.* 6:121–162.

KOHMAN, H. A., GODFREY, T. M., and ASHE, L. H. (1917). Manufacture of bread. U. S. Pat. 1,222,304, April 10.

KOHMAN, H. A., HOFFMAN, C., and BLAKE, A. E. (1915). Manufacture of bread. U. S. Pat. 1,158,933, Nov. 2.

——, ——, and GODFREY, T. M. (1915). Yeast-saving composition in bread-making. U. S. Pat. 1,151,526, Aug. 24.

KOSSEL, A. J. (1942). Über den Einfluss von Metallionen auf die Carboxylase. *Z. physiol. Chem.* 276:251–267.

KRIEBLE, V. K., SKAU, E. L., and LOVERING, E. W. (1927). Extraction of maltase from yeast. *J. Am. Chem. Soc.* 49:1728–1735.

LANDIS, Q. (1934). Diastatic activity in doughs and suspensions. *Cereal Chem.* 11: 24–35.

—— and FREY, C. N. (1943). Direct determination of fermentation rates in dough. *Cereal Chem.* 20:368–376.

LANNING, J. H. (1936). The effect of sucrose and maltose upon acid and gas production in dough. *Cereal Chem.* 13:690–697.

LARMOUR, R. K., and BERGSTEINSSON, H. N. (1936). Studies on experimental baking tests. III. The effect of various salts on gas-production. *Cereal Chem.* 13:410–420.

—— and BROCKINGTON, S. F. (1934). Studies on experimental baking tests. I. Effects of variation in baking formulas on gas production and loaf volume. *Cereal Chem.* 11:451–470.

LASNITZKI, A., and SZÖRÉNYI, E. (1935). The influence of alkali cations on the fermentation capacity of yeast. *Biochem J.* 29:580–587.

LECOURT, R. (1927). Recherches sur la toxicité de la farine de froment pour la levure. *Ann. brasserie dist.* 26:14–15, 72–77, 87–91, 102–107, 119–121, 134–137, 153–157.

LEIBOWITZ, J., and HESTRIN, S. (1939). The direct fermentation of maltose by yeast. *Enzymologia* 6:15–26.

—— and —— (1942). The direct fermentation of maltose by yeast. II. *Biochem. J.* 36:772–785.

LOHMANN, K., and SCHUSTER, P. (1938). Untersuchung über die Cocarboxylase. *Biochem. Z.* 294:188–214.

MICHAELIS, L., and MENTEN, M. L. (1913). Die Kinetik der Invertinwirkung *Biochem. Z.* 49:333–369.

MILLER, H., EDGAR, J., and WHITESIDE, A. G. O. (1943). An automatic gas recording apparatus. *Cereal Chem.* 20:355–361.

MUNZ, E., and BAILEY, C. H. (1936). Relation of amylase activity to gassing rate. *Cereal Chem.* 13:427–436.

OFELT, C. W., and SANDSTEDT, R. M. (1940). An apparent relationship between protein content and rate of proof. *Cereal Chem.* 17:707–713.

RICE, W. (1938). Sugars in bread. *Cereal Chem.* 15:672–677.

ST. JOHN, J. L., and BAILEY, C. H. (1929). Effect of dry skimmilk on the fermentation and hydrogen-ion concentration of doughs. *Cereal Chem.* 6:51–59.

SANDSTEDT, R. M., and BLISH, M. J. (1938). Maltose fermentation activators as affecting baking. *Cereal Chem.* 15:788–794.

SCHULTZ, A. S., and ATKIN, L. (1939). Fermentation of maltose. *J. Am. Chem. Soc.* 61:291–294.

——, ——, and FREY, C. N. (1937). A fermentation test for vitamin B_1. *J. Am. Chem. Soc.* 59:948–949.

——, ——, and —— (1937a). A fermentation test for vitamin B_1. II. *J. Am. Chem. Soc.* 59:2457–2460.

——, ——, and —— (1938). Influence of nicotinic acid on the fermentation method for vitamin B_1 determination. *J. Am. Chem. Soc.* 60:1514–1515.

——, ——, and —— (1939). Vitamin B_6, a growth promoting factor for yeast. *J. Am. Chem. Soc.* 61:1931.

——, ——, and —— (1939a). The vitamin B_1 content of wheat, flour, and bread. *Cereal Chem.* 16:643–648.

SCHULTZ, A. S., ATKIN, L., and FREY, C. N. (1939b). Fermentation of maltose in the dough. *Cereal Chem.* 16:648–651.

——, ——, and —— (1940). Influence of oxygen on the fermentation of maltose and galactose. *J. Am. Chem. Soc.* 62:2271–2272.

——, FISHER, R. A., ATKIN, L., and FREY, C. N. (1943). Sugar determination in starch hydrolyzates by yeast fermentation and chemical means. *Ind. Eng. Chem., Anal. Ed.* 15:496–498.

—— and KIRBY, G. W. (1933). Biological method for the determination of different sugars in starch degradation products. *Cereal Chem.* 10:149–155.

SHUTT, F. T. (1908). A chemical study of the grain and flour of the grades of wheat—1907. *Canada Dept. Agr., Central Exptl. Farm, Bull.* No. 60, Part II.

SIMPSON, A. G. (1936). The mechanism of dough fermentation: Note on a method for counting yeast cells in a fermenting dough. *Cereal Chem.* 13:50–54.

—— (1936a). The mechanism of gas production in dough fermentation. *Cereal Chem.* 13:140–152.

SKOVHOLT, O., and BAILEY, C. H. (1937). The effect of milk solids on fermentation reactions. *Cereal Chem.* 14:108–120.

SLATOR, A. (1906). Studies in fermentation. I. The chemical dynamics of alcoholic fermentation by yeast. *J. Chem. Soc.* 89:128–142.

—— (1908). Studies in fermentation. Part II. The mechanism of alcoholic fermentation. *J. Chem. Soc.* 93:217–242.

SMYTHE, C. V. (1939). The effect of certain tissue extracts, of ammonia salts and of certain amides on the rate of fermentation by bakers' yeast. *Enzymologia* 6:9–14.

SOBOTKA, H., and HOLZMAN, M. (1934). Direct fermentation of maltose. *Biochem. J.* 28:734–739.

—— and REINER, M. (1930). Selective fermentation. I. Alcoholic fermentation of glucose, fructose and mannose mixtures. *Biochem. J.* 24:926–931.

STONE, W. E. (1896). The carbohydrates of wheat, maize, flour, and bread. *U. S. Dept. Agr. Official Expt. Sta. Bull.* No. 34:7–28.

STUART, L. S., and HARRIS, T. H. (1942). Bactericidal and fungicidal properties of a crystalline protein isolated from unbleached wheat flour. *Cereal Chem.* 19:288–300.

SWANSON, C. O. (1912). Acidity in wheat flour; its relation to phosphorus and to other constituents. *J. Ind. Eng. Chem.* 4:274–278.

TANRET, M. C. (1891). Sur la lévosine, nouveau principe immédiat de quelques céréales. *Bull. soc. chim.* 5:724–731.

TELLER, G. L. (1912). The carbohydrates of wheat and wheat products and changes in same during development of the grain. *Proc. 8th Intern. Congr. Pure Applied Chem., Sec. VIa,* 13:273–278.

THORNE, R. S. W. (1933). The nitrogenous metabolism of yeast. Part III. The ability of certain pure chemical compounds to function as nitrogen sources for yeast. *J. Inst. Brewing* 39:608–621.

TRAUTWEIN, K., and WASSERMANN, J. (1931). Die pH-Empfindlichkeit der atmenden und gärenden Bierhefe. Umschaltung von Gärung auf Atmung. *Biochem. Z.* 236:35–53.

TURLEY, H. E. (1924). Counting yeast cells in dough. *Cereal Chem.* 1:261–267.

VAN NIEL, C. B., and ANDERSON, E. H. (1941). On the occurrence of fermentation assimilation. *J. Cellular Comp. Physiol.* 17:49–56.

WIDDOWSON, E. M. (1941). Phytic acid and the preparation of food. *Nature* 148:219–220.

WILLARD, J. T., and SWANSON, C. O. (1913). The baking qualities of flour as influenced by certain chemical substances, milling by-products and germination of the wheat. *Agr. Expt. Sta. Kansas Bull.* No. 190.

WILLSTÄTTER, R., and BAMANN, E. (1926). Zur Kenntnis der Hefemaltase. Sechste Mitteilung. *Z. physiol. Chem.* 151:242–272.

—— and ROHDEWALD, M. (1937). Über die erste Phase der Gärung durch Hefe. *Z. physiol. Chem.* 247:269–280.

—— and STEIBELT, W. (1920). Bestimmung der Maltase in der Hefe. *Z. physiol. Chem.* 111:157–170.

ZELLER, H. (1926). Wirkung von Ammonsalzen auf die Hefegärung. IV. *Biochem. Z.* 175:135–161.

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